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THE UNIVERSITY OF ALBERTA

SOME ADENOSINETRIPHOSPHATASE ACTIVITIES

IN RAT MYOMETRIAL TISSUE

by

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The undersigned certify that they have read, and
recommend to the Faculty of Graduate Studies for accep-
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ments for the degree of Doctor of Philosophy.

ABSTRACT

The ATPase activities of various preparations of rat myometrium have been studied. These preparations are; nuclear mitochondrial and microsomal fractions isolated by differential ultracentrifugation; a plasma membrane preparation, total homogenate and intact tissue.

All preparations examined possessed an ATPase activity which required a divalent cation for activation. In the mitochondrial and microsomal preparations, the optimal Mg^{++}/ATP ratio was 1/1. Broad pH activity curves were shown for these two fractions as well as the total homogenate. The substrate specificity with Mg^{++} was tested for total homogenate, microsomal and mitochondrial fractions and found to be very low.

Under the conditions of study, the cardiac glycoside, ouabain, was shown to slightly inhibit the Mg ATPase activities of the microsomal fraction, membrane preparation and total homogenate, but not the activity of the intact tissue. These results suggest that the inhibitory site of action of ouabain in this tissue may be on the inside of the cell.

The microsomal fraction was studied in more detail because of the possible presence of an enzyme involved in

active cation transport.

No Na^+ or K^+ activation of ATPase could be found in freshly isolated microsomal material. However, various treatments produced Na^+ activation (maximum about 60%). In no case was K^+ activation, alone or with Na^+ demonstrable.

Ca^{++} in the presence of Mg^{++}, and ADP were both inhibitors of the microsomal Mg ATPase activity.

Na^+ activation was also demonstrable in the total homogenate and membrane preparation. In both cases the Na^+ activation seemed to be roughly equivalent to ouabain inhibition.

In fresh microsomes, ouabain caused a 10 to 30% inhibition of ATPase activity in the absence of Na^+ or K^+. The Na^+ activation found in stored fractions was only partially inhibited by ouabain.

The presence of a Na^+ activated, K^+ independent ATPase in rat myometrium may be consistent with the nature of the active transport mechanisms in this tissue as revealed by other studies of ion fluxes and net ion movements.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

1. ATPase = adenosinetriphosphatase

2. Ca, Mg or Zn ATPase = adenosinetriphosphatase activity in the presence of the designated divalent cation.

3. $Na^+ + K^+ - Mg^{++}$ ATPase = ATPase activity requiring Na^+ and K^+ for maximum activity.

4. $Na^+ - Mg$ ATPase = ATPase requiring Na^+ for maximum activity.

5. ATP = adenosinetriphosphate

6. ADP = adenosinediphosphate

7. AMP = adenosinemonophosphate

8. CP = creatine phosphate

9. CTP = cytosinetriphosphate

10. DNFB = dinitrofluorobenzene

11. DNP = 2,4-dinitrophenol

12. DOC = desoxycholate (deoxycholate)

13. FS = Fiske-Subbarow method of phosphate determination

14. GTP = guanosinetriphosphate

15. IAA = iodoacetic acid

16. ITP = inosinetriphosphate

17. LDH = lactic dehydrogenase

18. LI = LeCocq-Inesi method of phosphate determination

19. NEM = N-ethyl maleimide

20. NTP = nucleosidetriphosphate

21. NTPase = nucleosidetriphosphatase

22. PCMB = para-chloromercuribenzoate

23. P_i = inorganic orthophosphate

24. TCA = trichloroacetic acid

25. Tris = Tris (hydroxymethyl) aminomethane

26. UDPG = uridinediphosphoglucose

27. UTP = uridinetriphosphate

INTRODUCTION

I. Background Leading to the Study

It has been known for many years that cells are able
to maintain a high potassium and a low sodium content
internally, in spite of high sodium low potassium content
externally, and many workers have postulated a cyclic
coupled transport system to explain the tendency for these
ions to move reciprocally (Dunham and Glynn, 1961; Glynn,
1957; Hodgkin, 1958). According to this hypothesis, sodium
transport outward, and potassium transport inward were
coupled, and required a source of energy, since sodium, and
in some cases, potassium as well moved against their respec-
tive electrochemical gradients. Ussing (1960) defined this
as active transport. An oversimplified mechanism, suitable
at this point, follows. A more detailed mechanism will be
found in a later section of this thesis.
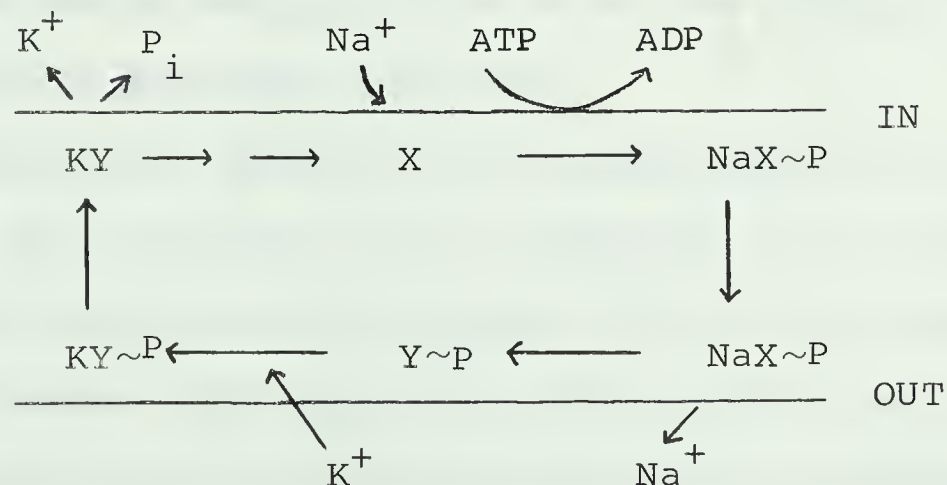
X = a Na^+ carrier molecule which can be converted to

Y = a K^+ carrier molecule.

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It has been shown by experiments on nerve (Caldwell, 1960) and red blood cell ghosts (Whittam, 1958; Hoffman, 1960) that the energy for active transport is derived from ATP, and no other nucleotide can replace this source.

In 1948 Libet and 1954 Abood and Gerard demonstrated that nerve homogenates contained an ATPase activity, but it was not until 1957 that Skou isolated an ATPase enzyme system from crab nerve microsomal fractions, which possessed properties indicating that it might be a component of the active transport system, in that in addition to magnesium, it required both sodium and potassium for maximum activation.

In 1953 Schatzmann showed that the cardiac glycoside, ouabain, inhibited active cation transport in red blood cells, and Skou, in 1960 showed that this glycoside inhibited the sodium and potassium stimulated ATPase activity of crab nerve microsomal fractions.

Daniel and Robinson (to be published) have shown that ouabain also inhibits active transport in the rat uterus, but the resulting ion movements differ from those found in other tissues. Although all tissues show a net gain of sodium and loss of potassium upon active transport inhibition, in rat uterus this is effected chiefly by an increased sodium influx and an increased potassium efflux, not by a decreased potassium influx and decreased sodium

efflux as occurs in other tissue. Due to these unusual ion movements, it was of interest to study the ATPase system of rat uterus.

II. Energy Metabolism of the Uterus

A. High Energy Phosphate Content and Aerobic Glycolysis

ATPase is involved in energy metabolism. Therefore a summary of energy production and utilization in the uterus will follow.

It has been shown that ATP and CP are the major energy sources for muscular contraction in both skeletal and smooth muscle (Davis, 1965; Needham, 1962). However, levels of these compounds in the uterus are far below those found in skeletal muscle (Walaas and Walaas, 1950a,b), even though estrogen can significantly increase their content in the myo-metrium (the myometrium is the smooth muscle of the uterus). The high energy content of uterus has recently been examined by Kumar et al (1962) who have shown that the human myo-metrium contains only about 15-20% of the high energy com-pounds of the frog rectus abdominis. Daniel and co-workers (unpublished) have shown that the high energy phosphate content (ATP + ADP) of rat myometrium is less than 2 micro moles per gram wet tissue weight with ATP about 1 μmole, compared to values for skeletal muscle ATP ranging from 3 to 7 μmoles/gm tissue for ATP alone.

Walaas and Walaas (1950a) have suggested that this high energy phosphate difference may be in part the reason for the different types of contraction found in the two different types of muscles, although more recent work with protein models from uterus suggest that the protein content and myofibrillar ATPase activity and cellular arrangement of these myofibrils also play a significant role.

The metabolic processes which yield ATP as a final product are still not understood for the myometrium. In 1952 Walaas et al showed that rat myometrium undergoes aerobic glycolysis, i.e., despite the presence of abundant oxygen, uterus still accumulates lactic acid. The only other tissues known to exhibit this phenomenon are retina, kidney medulla, gastric mucosa, and tumor cells (Cantarow and Shepartz, 1962). This may correlate with the above mentioned fact that high energy phosphate compounds are lower in myometrium than in skeletal muscle, or it may be that energy metabolism is less efficient in the myometrium.

Let the high energy metabolism be represented schematically as follows.

$$\text{production} \xrightarrow{1} \text{P} \xrightarrow{2} \text{utilization}$$

Let step one consist of both substrate and oxidative phosphorylation, and let step two consist of all energy utilizing reactions, such as contraction, active transport

or synthetic processes.

Since uterine myometrium contains fewer high energy
compounds than skeletal muscle, either step one is slower,
or step two is faster in the former. Thus either an in-
creased reaction rate of step two or decrease in step one
could result in lower content of high energy compounds.
It is now obvious that both steps one and two must be
studied in detail before any conclusions can be reached
concerning the relative efficiency of the energy metabol-
ism of uterine muscle compared to that of other contrac-
tile tissue.

The idea of lower energy content of smooth muscle
had been suggested earlier by Csapo and Gergely (1950)
who showed that skeletal muscle poisoned with IAA under
N_2 continued to contract for 70 times, while uterine
tissue under these same conditions contracted only 10
times. They also found that the ratio of ATP in skele-
tal muscle compared to that of uterine muscle was about
6.6 to 1, thus demonstrating a good correlation between
this and the ratio of the number of contractions, and indi-
cating that approximately the same amount of energy was
required for a single contraction in each muscle type.

Kerly (1937) demonstrated in the rat that both
oxygen consumption and anaerobic glycolysis are highest
during follicular maturation (pro-estrus) and lowest at

the end of estrus. Aerobic glycolysis varied in the oppo-
site sense, and was lowest during pro-estrus and highest
immediately after estrus. This same worker also showed
that the amount of anaerobic glycolysis per mg. dry weight
of uterus per hour exceeded aerobic glycolysis at all times
of the cycle, indicating the presence in this tissue of a
continual Pasteur effect.

B. Myometrial Mitochondria

Mitochondria are found in this tissue, and the over-
all cytology of the cell seems to differ only quantitatively
from skeletal muscle (Schoenberg, 1958). However, Wakid
and Needham (1960) were unable to demonstrate any oxidative
phosphorylation in mitochondria isolated from rat uterus,
and Gautheron et al (1961) demonstrated low respiration
in mitochondria of guinea pig uterus. Thus, perhaps lactic
acid accumulation may be due to mitochondria differing in
function or efficiency from those of other tissues.

Gautheron and co-workers (1961), and Volfin et al
(1957) have noted interesting effects of adrenaline on the
high energy phosphate levels in rat uterus. They showed
that besides an inhibitory effect of the hormone on endo-
genous oxygen uptake and anaerobic glycolysis, adrenaline
also promoted a large increase in inorganic phosphate and
decrease in high energy phosphates. Both inhibitory meta-

bolic effects of this compound can be overcome by DNP.

C. Glycogen Synthesis

Another aspect of metabolism which may be different in uterine tissue compared to other tissue is the mechanism of glycogen synthesis. The concentration of glycogen in the rabbit uterus has been shown to be greatest following estrogen treatment, and is localized in both the endometrium and myometrium (Reynolds, 1949, 1951). In most tissues glycogen is synthesized from UDPG by the action of UDPG-glycogen synthetase. It has been considered that the glycogen synthesizing enzyme in uterus is also UDPG-glycogen synthetase, and that phosphorylase is concerned with glycogen breakdown. However, Bo and co-workers (1964a,b) were only able to demonstrate the presence of the former enzyme in the uterus of ovariectomized animals (both treated with estradiol and untreated) in the outer longitudinal muscle of the myometrium, while the latter enzyme was present in both the longitudinal and the circular muscle. These data, along with the earlier histochemical demonstration of glycogen synthesized from glucose-1-phosphate suggests that the glycogen in uterine tissue may not all be synthesized through the UDPG pathway, but might also be synthesized from the hexose sugar by the action of phosphorylase.

The question of glycogen synthesis in myometrium remains unsolved. Leonard and co-workers (1963) and Schare et al (1965) demonstrated that rat uterus contains a phosphorylase stimulated by adrenaline, as have Diamond and Brody (1965, 1966).

Scott and Lisi (1960) showed that estrogen adminis- tration caused an increase in total phosphorylase and gly- cogen. A possible explanation of this observation aside from the regulation of glycogen synthesis by phosphorylase may be found in the demonstration of Gorski and Mueller (1963) that estradiol administration to rats produces sub- stantial increments in the content of UTP and UDPG in the uterus, and that despite increased phosphorylase activity coincident with increased glycogen content, the UDP system is still quantitatively the chief source of glycogen syn- thesis.

D. Lactic Dehydrogenase

Lactic dehydrogenase reduces pyruvic acid to lactic acid, and is therefore important in energy metabolism. It has been shown by Allen (1961) and Goodfriend and Kaplan (1964) that the enzyme consists of various subunits.

Different proportions of H (heart type) and M (muscle type) subunits result in the enzyme having different pro-

perties. The susceptibility of heart LDH to inhibition by pyruvate is compatible with the adaption of the heart to aerobic metabolism of pyruvate and NADH. Skeletal muscle enzyme (M subunits) however, is not inhibited by pyruvate, and thus enables the tissue to derive energy from anaerobic pathways when oxygen is limited and pyruvate accumulates.

Biron (1964) has shown that pregnancy as well as estradiol administration alters the isoenzyme distribution in the rat and rabbit uterus, by increasing the proportion of M type subunits. This leads one to conclude that in the pregnant state, enzymatic alterations occur which prepare the tissue for anaerobic metabolism.

E. Effects of Estrogen Administration on Enzyme and ATP Levels

Knox, in his review (1956), tabulated the effects of estrogen administration on enzyme activities in the uteri of different animals. No reference was made to respiratory enzymes except for the work of Telfer (1953) who showed that administration of 5 micrograms of estradiol to the ovari- ectomized rat increased the succinate and ascorbate Q_{O_2} (succinoxidase, cytochrome oxidase) and promoted extensive RNA synthesis, while having no effect on DNA synthesis. This finding has more recently been expanded to include protein synthesis.

Quite recently Aaronson et al (1965) have shown that the ATP level in the uterus decreases at an early stage of estrogen administration, and that this decrease could be prevented by the administration of Actinomycin D. They further showed that ATP levels seemed to bear an inverse relation to the reported rates of RNA synthesis, thus implying that RNA synthesis and not protein synthesis is the more demanding utilizer of ATP in uterine tissue.

It was demonstrated by Gorski and Mueller (1965) that estrogen treatment had little effect on ATP levels. This finding conflicts with the work cited above, with the work of Volfin et al (1957) who found an ATP increase, and with the hypothesis of Villee et al (1960) who have postulated that the activation of a specific soluble transhydrogenase by estrogen would increase the production of high energy phosphate compounds and promote the anabolic reactions necessary for growth of the uterus. Gorski and Mueller also found a large increase in the uridine nucleotides.

Gross (1961) and Thiery and Willihagen (1963) also reviewed uterine enzymes which undergo cyclic variations. Those involved in energy metabolism were alkaline phosphatases of the endometrium, DPN oxidase, myofibrillar ATPase and hexokinase. Acid phosphatases, succinoxidase and LDH are said to be under the influence of progesterone.

The recent finding of Goodall (1965) that estradiol initiates and progesterone inhibits the active synthesis of degradative enzymes such as proteolytic enzymes, aldolase and RNAase, is as yet not fully explained. He suggests that since progesterone is needed to maintain active uterine growth, progesterone could prevent the degradative activity of these enzymes and that the absence of this hormone results in activation of the degradative enzymes, leading to tissue involution.

In summary we can say that uterine tissue exhibits a biochemical profile which shows much variability from many other "standard" tissues. Before any conclusions can be reached about the metabolism and functioning of this organ as a whole, hormonal influences on all the topics covered thus far will have to be determined. It is essential that the energy content of the tissue under various stages of treatment must be analyzed to clarify the picture of energy metabolism.

III. Mitochondria and Mitochondrial ATPase

No review of energy metabolism of a tissue would be complete without reference to the role of mitochondria. Because of the great quantity of work done on structure and function of this organelle, great detail will not be given in this review. For extensive information, the

reader is referred to the two books edited by Chance and his colleagues (1963, 1965), and the book written by Lehninger (1964).

The primary function of the mitochondrion is to couple the synthesis of ATP (from ADP and P_i) to the aerobic oxidation of some metabolite. In animal tissue the principal substances which undergo oxidation are the intermediates of the citric acid cycle. In most mitochondria which carry out the citric acid cycle, a variety of substances other than the actual intermediates of the cycle can also be oxidized. Examples are beta-hydroxybutyric acid, and alpha glycero-phosphate. It has been emphasized that the mitochondrion is a complete operational unit in that it contains all the constituents necessary to carry out its functions. Only inorganic phosphate, ADP, molecular oxygen, substrate and Mg^{++} need be added to isolated mitochondria to achieve oxidative phosphorylation.

The respiratory chain sequence has been well delineated, and may be found in detail in the book by Lehninger (1964).

A. Origin of ATPase Activity

The ATPase activity of mitochondria is one of the partial reactions involving P_i and/or ATP which occur in the absence of net electron transfer and in the absence

of submitochondrial organization capable of oxidative phos-
phorylation. It may be the result of the occurrence of
reversible intermediate steps in the phosphorylation mechan-
ism (Racker, 1961). This hydrolytic activity is stimulated
by DNP and other uncoupling agents in both intact mitochon-
dria and in phosphorylating sub mitocondrial particles, and
is possibly due to abnormal hydrolysis of some
intermediate in the coupling mechanism. If a high energy
or low energy intermediate is actually hydrolyzed by DNP,
thus causing increased ATPase activity, this could conceiv-
ably be the mechanism of uncoupling by DNP and related com-
pounds. At present this is in dispute.

(i) DNP and Mg^{++}Effects

Aged mitochondria exhibit ATPase activity upon the
addition of Mg^{++}. If intact, fresh mitochondria are
treated with DNP they exhibit ATPase activity which is
independent of Mg^{++}. There has been much discussion as
to whether DNP and Mg^{++}-stimulated ATPase are different
entities, and no conclusions have been reached. Racker
(1961, 1965) feels that since all results can be explained
with one enzyme, there is no need to involve a two enzyme
system.

The first suggestion that the ATPase activity of mito-
chondria might represent a partial reversal of oxidative
phosphorylation was presented by Lardy and Elvejhem(1945).

It was proposed at that time that the uncoupling by DNP was due to the breakdown of an intermediate, releasing P_i and accounting for the ATPase activity stimulated by DNP. It was also demonstrated that Mg was required for maximum activity and that ADP inhibited the enzyme, the latter effect perhaps being responsible for the latency of the ATPase due to the presence of ADP in the mitochondria. The inhibitory effect of ADP on mitochondrial ATPase was demonstrated by Gatt and Racker (1959a,b) who continuously removed ADP with phospho-enol pyruvate and pyruvate kinase, and observed a five fold stimulation of the activity in freshly isolated liver mitochondria.

The recent report of Hemker (1964) of ATPase inhibition by high concentrations of DNP supports the earlier work of Myers and Slater (1957). This finding could indicate that this and other phenols might be acting at two separate sites, both of which are involved in oxidative phosphorylation. Hemker suggests two mechanisms, both of which could result in ATPase activity.

1) An uncoupling phenol at lower concentrations could allow oxidation to proceed without phosphorylation, leaving P_i available, and thus

leading to an ATPase activity.

2) An uncoupling phenol could react with a high
   energy intermediate to release P_i.

Again, it should be emphasized that the exact
mechanism of action of the uncoupling agents is not known.

B. Effect of Ions

The effects of ions on the mitochondrial ATPase has
been studied by Ulrich (1963, 1964a,b) and Pena-Diaz (1964).
The former has concluded that the inhibitory effect of mono-
valent ions is due to an electrostatic mechanism of action.
To arrive at the conclusion, Ulrich studied the effects of
amino acids and sugars on the ATPase activity, the reason
being that if the inhibition was due mainly to the charge
on the ion, sugars should have little effect, while the
amino acids' effect on the enzyme would depend largely on
the pH of the medium, regulating the charge of the amino
acid, and the nature of the acid itself. This prediction
held true, and at the same time, Ulrich succeeded in elim-
inating a complexing effect between the acids and magnesium
as a possible cause of inhibition.

Pena-Diaz et al (1964) have shown that the response
of the ATPase system to sodium and potassium seems to
depend on the structural condition of the mitochondria,
since when they were disrupted or altered by sonication or

desoxycholate, a greater stimulatory activity was measured in the presence of potassium. Thus the latter workers have shown that sodium and potassium stimulate the ATPase activity after appropriate pretreatment, contrary to Ulrich. To date, no reconciliation of these opposing data, both obtained from rat liver mitochondria, has appeared.

Almost no studies of mitochondrial function in the uterus have been published, except for the work noted earlier of Wakid and Needham (1960) and Gautheron and co-workers. The presence of aerobic glycolysis and the predominance of LDH of the M type should offer interesting biochemical studies.

C. Solubilization of Mitochondrial ATPase

In the past five years, Racker and his associates have isolated a soluble ATPase activity which appears to be an integral part of the oxidative phosphorylation process. This is the only mitochondrial ATPase activity which has been solubilized. This activity is cold labile, and insensitive to oligomycin, an inhibitor of oxidative phosphorylation. These properties may be reversed by the addition of various other soluble fractions obtained from the mitochondria treated with trypsin and urea (Racker, 1965).

IV. Actomyosin ATPase

Actomyosin has been established for many years as
the contractile protein in both skeletal muscle (Perry,
1961; Kielly, 1964) and smooth muscle (Needham, 1962,
1964a,b). The energy needed for the contraction is most
probably gained by the splitting of ATP by actomyosin
ATPase (Davies,1965) although there still seems to be
some dispute about this fact.

The enzymatic activity of actomyosin ATPase is
highly dependent on the prevailing ionic conditions
(Perry, 1961; Kielley, 1964). Under suitable conditions,
actomyosin in water forms a gel which contracts strongly
upon the addition of ATP. This superprecipitation is
thought by many to be analogous to contraction as it
occurs in intact muscle. For many years it has been
known (Needham, 1964b) that uterine smooth muscle con-
tains a protein closely resembling skeletal muscle acto-
myosin. Furthermore, skeletal muscle myosin will react
normally with uterine actin to give the highly viscous
actomyosin, and similarly uterine myosin with skeleteal
actin. One large difference exists between skeletal
muscle and smooth muscle (Needham, 1962) and that is
that the latter possesses only 6-10 mg. actomyosin pro-
tein per gram wet tissue weight, compared to about
70 for the former. It is worth noting the similarity

between this ratio and the ratios quoted earlier in this
introduction (page 5) for the number of contractions and
the content of ATP. One could make many speculations from
these figures, although that is beyond the scope of this
work.

Of the two isolated components of actomyosin, actin
and myosin, only the latter has ATPase activity. A major
difference between myosin and actomyosin ATPase is that
magnesium in small quantities strongly inhibits calcium
activated myosin ATPase, but not actomyosin ATPase (Szent-
Györgyi, 1960). It has been shown (Carvalho, 1966) that
zinc can substitute for magnesium and cause both actomyo-
sin ATPase activation and superprecipitation.

Szent-Györgyi (1960) and others have shown that when
skeletal muscle myosin is treated with trypsin, the protein
is split into heavy meromyosin (HMM, M.W. 350,000) and
light meromyosin (LMM, M.W. 150,000). HMM is water soluble
and contains all the ATPase activity, while LMM retains
the globular properties of myosin. Smooth muscle myosin
behaves similarly with trypsin treatment, and the resulting
LMM and HMM have similar properties in so far as they have
been studied (Needham, 1964). The protein, tropomyosin B,
while demonstrable in skeletal muscle and invertebrate
smooth muscle, has never been isolated from uterine smooth
muscle, while tropomyosin A is present in all three.

A major difference between uterine and skeletal muscle ATPase activities is shown with the following table (Needham, 1962).

	μmoles P set free per mg. N per hour
From Skeletal Muscle	
Actomyosin (Ca^{++} or Mg^{++} activated)	4.5
Acto-H-meromyosin (HMM) (Ca^{++} activated)	9.9
From Uterine Muscle	
Actomyosin (Ca^{++} activated)	0.5
Acto-H-meromyosin (Ca^{++} activated)	5.5

A. Effects of DNP

The effects of DNP on the Ca^{++} activated myosin ATPase have been studied extensively by Chappell and Perry (1955) who demonstrated that DNP stimulated myosin ATPase as well as mitochondrial ATPase. The stimulating effect diminished progressively with the addition of actin, but the ATPase of intact fibers was still sensitive. Since DNP is an uncoupler of oxidative phosphorylation, it is interesting to speculate on this compound's mode of action on the myosin ATPase.

Although much work has been done on myosin ATPase and its role in contraction, the actual chemical and molecular mechanism of this phenomenon is still not clear (Davies, 1963). The irregularity of the myofibrillar arrangement in smooth muscle compared to that of skeletal muscle (Lowy and Hanson, 1962) leaves much work to be done on the actual mechanism of contraction in the non striated tissue as well.

V. ATPase of the Endoplasmic Reticulum, Associated with Vesicular Ca Uptake and the Relaxation of Muscle

In 1948 and 1950 Kielley and Meyerhof reported the isolation from rat hind limb muscle of a magnesium stimulated ATPase associated with particulate matter, which was inhibited by Ca^{++}. This enzyme was easily distinguishable from actomyosin ATPase since the latter was not magnesium activated, and not inhibited by Ca^{++} and was not associated with particulate matter. Its importance was a matter of speculation until Marsh (1952) and Bendall (1953) made important contributions to the idea of active muscle relaxation, and directly implicated the ATPase mentioned above. By active muscle relaxation it is meant that the relaxing process is unique process in itself, with a separate mechanism, and is not merely the result of the passive ending of a contraction.

While it has been generally accepted for many years
that the contractions of skeletal muscles, actomyosin and
glycerinated muscle fibers require Ca^{++} in addition to
magnesium and ATP, it has only recently been suggested
that the relaxing effect of chelating agents and skeletal
muscle microsomes can be explained by their ability to
lower the Ca^{++} concentration of the test system below 10^{-7}
M in the presence of 5 mM ATP and Mg^{++}. This concentration
of calcium is the critical value below which actomyosin
ATPase activation and protein contraction are blocked by
physiological concentrations of magnesium. The microsomes
accomplish this by a powerful uphill transport system for
Ca^{++} which operates in the presence of ATP and magnesium.

For some years, workers suggested that the synthesis
of a soluble relaxing factor isolatable from skeletal
muscle microsomes was responsible for the active relaxation
of muscle (Briggs and Fuchs, 1960; Muscatello et al, 1962).
This concept, although not completely abandoned, has fallen in-
to disrepute among workers in the field. For a complete
analysis of the subject, the reader is referred to the
papers of Gergely, Franzini-Armstrong, Weber and many others
in the symposium (1964).

The actual mechanism of the Ca^{++} uptake process by
microsomal vesicles is not known, although the current
suggestion is that the uptake is due to Ca^{++} binding to

specific receptor sites made accessible to Ca^{++} by ATP and Mg^{++} and subsequent active transport. The possibility that ATP itself could function as a carrier has been eliminated (Hasselbach, 1964).

According to Hasselbach (1964) the only energy source utilizable for active transport of Ca^{++} by the endoplasmic reticulum (the in vivo form of the isolated microsomal fraction of skeletal muscle (Huxley, 1965)) is ATP. Thus an ATPase activity is both postulated and demonstrable here, which requires magnesium, and is activated by Ca^{++}. This is the extra ATPase activity, and it is responsible for the active uptake Ca^{++} uptake. This activity is activated by Ca^{++} in the presence of oxalate. The basic ATPase activity (the initial microsomal activity, mentioned earlier), is inhibited by Ca^{++}, and no active process is associated with it. The fact that the Ca^{++} uptake and extra ATPase splitting can be well correlated suggests that the energy of ATP hydrolysis is used for transporting Ca^{++} through the vesicular membranes.

To date little work has been done on the vesicular ATPase involved in Ca^{++} uptake and the relaxation of smooth muscle. Batra and Daniel (unpublished observations) have been unable to demonstrate active Ca^{++} uptake in microsomal fractions of rat uterus. Hasselbach was also unable to demonstrate such activity in cow uterus (1964).

Suggestions have been made for cardiac muscle that mitochondria as well as endoplasmic reticulum are involved in the relaxing effect. Furthermore, Honig and Stamm (Symposium, 1964) suggest that cardiac muscle contains two separate relaxation systems, one the particulate material per se, and the other a soluble substance elaborated by the reticulum, requiring ATPase activity for its synthesis. A complete review of relaxing factor and muscle relaxation has recently been published (Hasselbach, 1964).

VI. <u>ATPase Associated with the Cell Membrane and Involved</u> <u>with Active Cation Transport</u>

The final ATPase to be discussed will be that associated with the cell membrane and believed to be involved with active cation transport across this barrier. Active transport is defined as the net movement of a substance against its electrochemical gradient when coupling of bulk flow to solute has been eliminated. An analysis of the experimental work done to establish the existence of active transport would be too extensive to be presented here. Since intracellular Na^+ concentration is maintained at a low level compared to the extracellular, and since Na^+ diffuses continuously into the cell in response to both electrical and chemical gradients, work must be done continuously by the cell to expel Na^+.

The justification for including an ATPase in any mechanism of active transport has been well documented, however it would be profitable to review these criteria as originally set down by Post et al (1960) and elaborated upon more recently by Skou (1965).

The former group isolated an ATPase from broken human red blood cell membranes, which had the following characteristics in common with the active cation transport system of intact erythrocytes.

1) Both systems were located in the membrane.

2) Both systems use ATP, and not ITP.

3) Both systems require Na^+ and K^+ together.

4) Concentration for half maximal activation by K^+ in the presence of Na^+ was 3 mM for the enzyme and 2.1 mM for transport by the intact erythrocyte.

5) Concentration for half maximal activation by Na^+ in the presence of K^+ was 24 mM for the enzyme and 20 mM for the intact erythrocyte.

6) In both preparations, K^+ activation was competitively inhibited by Na^+.

7) Concentration for half maximal inhibition by ouabain in the presence of Na^+ and K^+ was 10^{-7} for the enzyme, and 5-7 10^{-8} M for transport in the erythrocyte.

8) NH_4^+ ion could substitute for K^+ ion but not for Na^+ ion in both systems.

More recently, Skou (1965) has elaborated on these and other observations, and has arrived at a list of eight general requirements of a Na^+ transport system.

1) It should be located in the cell membrane.

2) It should have an affinity for Na^+ that is higher than for K^+ at a site located on the inside of the cell membrane.

3) It should have an affinity for K^+ that is higher than for Na^+ at a site located on the outside of the membrane.

4) It should contain an enzyme system that can catalyze the hydrolysis of ATP and thus convert the energy for ATP into a movement of cations.

5) It should be capable of hydrolyzing ATP at a rate dependent on concentration of Na^+ inside the cell, and K^+ outside the cell.

6) It should be found in all cells that contain an active linked transport of Na^+ and K^+.

7) It should be inhibited by cardiac glycosides to the same extent as the intact transport system.

8) It should have the same quantitative relation to

Na^+ and K^+ as the transport system in the intact
cell.

In 1961, 1962 and 1963, Bonting and co-workers re-
ported on the presence of Na^+, K^+ activated ATPase in the
total homogenates of many different tissues. Although
these workers studied many tissues systematically, it is
important to note that in only one study (Bonting, 1963)
was any attempt made at differential centrifugation, to
attempt to localize the activity. In many case since
then, the tissue examined by these workers have been sub-
jected to a more thorough analysis, for the specific
localization of the transport ATPase. For a complete
listing, the review articles of Skou (1964, 1965) and
Judah and Ahmed (1964) should be consulted.

A. Mechanism of ATP Hydrolysis, and the Existence of
a Phosphorylated Intermediate

In 1960, Skou expanded on the work he had done in
1957 on crab nerve ATPase and postulated a sequence of
events.

$$E + ATP + 2Mg^{++} + nNa^+ + mK^+ \rightleftharpoons \begin{matrix} mK \\ EMg_2ATP \\ nNa \end{matrix}$$

$$\begin{matrix} mK \\ EMg_2ATP \\ nNa \end{matrix} \rightleftharpoons \begin{matrix} mK \\ E \sim P \\ nNa \end{matrix} + 2Mg^{++} + 2ADP$$

$$\begin{matrix} mK \\ E \sim P \\ nNa \end{matrix} \longrightarrow E + Pi + nNa^+ + mK^+$$
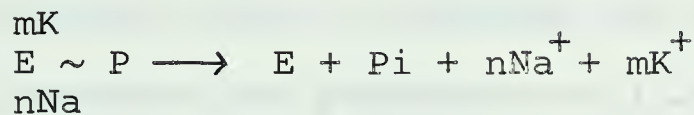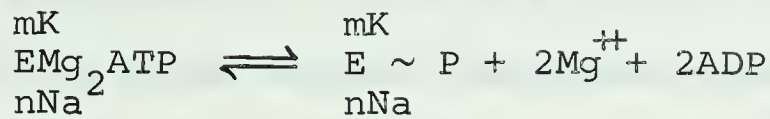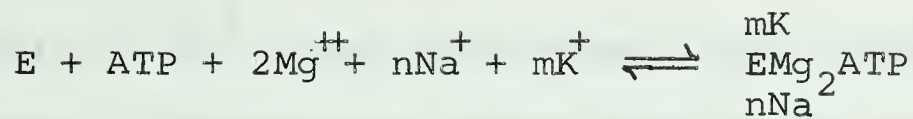
where E = enzyme

He also demonstrated a competitive inhibition between Na^+ and K^+. When Mg^{++} and Na^+ were present in the system, K^+ exerted two different actions on the enzyme activity. In lower concentrations it showed a stimulation, and higher concentration, an inhibition, indicating that the point of K^+ inhibition was the same as Na^+ activation, since high concentration of K^+ decreased the activity to that seen with just Mg^{++} present.

In 1962, Ahmed and Judah, using P^{32} labelling techniques, showed that rapid decreases in phosphoprotein and ATP label occurred when liver slices were incubated in choline Ringer P^{32} solution as compared to incubation in Na Ringer with P^{32}. The change in the phosphoprotein label always preceded that of ATP. The addition of small

amounts of Na^+ (less than 40 mM) could reverse these effects. Furthermore, drugs such as Benadryl, Phenergan, and Strophanthin, which are known to inhibit ion movements in liver slices, decreased phosphoprotein labelling. The addition of Na^+, however, in the presence of these inhibitors, did not increase the ATP label, although it did increase the phosphoprotein label, indicating that ATP could not be the sole Na^+ carrier, and that some other intermediate compound was involved. If ATP were the sole carrier of Na^+, then the above mentioned drugs would prevent the Na^+ stimulation of ATP turnover caused by the addition of Na^+ to slices in choline Ringer. It was suggested that Na^+ transport was mediated by at least two separate steps.

$$1) \quad ADP + P_i \underset{}{\overset{Na^+}{\rightleftharpoons}} ATP - Na$$

$$2) \quad ATP - Na + X \rightleftharpoons ADP + X \sim P_i + Na^+$$

$$3) \quad X \sim P_i + K^+ \longrightarrow X + K^+ + P_i$$
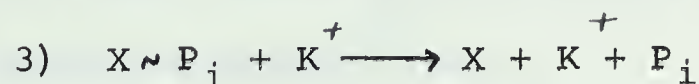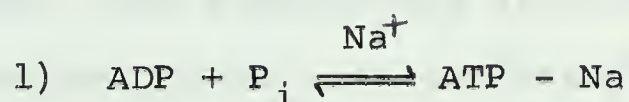
In 1963, Charnock et al, using ATP labelled with P^{32} only in the terminal position were able to show that ATP^{32} was hydrolyzed to ADP and P_i^{32} via a phosphorylated intermediate. Na^+, but not K^+ ions were required for the

formation of the intermediate and ADP and K^+ ions were sub-
sequently required for the dephosphorylation step with the
release of P_i^{32}.

Ouabain was without effect on the formation of this
intermediate complex, but it was able to completely inhibit
the K^+ requiring dephosphorylation step. Experiments also
showed that Na^+ ions were bound to the enzyme system during
the phosphorylation step, and were released when the inter-
mediate complex was destroyed.

Very similar results with Na^+ binding have also been
reported by Jarnefelt and von Stedingk (1963), who found
that the binding of Na^+ to brain microsomal particles was
dependent on the concentration of ATP and Na^+, and that it
was specific for ATP, as other nucleotides did not promote
binding. The binding was also inhibited by 5 mM Ca^{++}, and
the release of Na^+ was inhibited by 10^{-5} M ouabain. Post
and co-workers (1965) have more recently expanded their
work on the phosphorylated intermediate of kidney membranes.
Walz and Chan (1966) have reported Na^+ binding to erythro-
cyte membrane preparations.

With regard to the Na^+ binding mentioned above, it
should be noted that Ahmed and Judah (1966) very recently
have shown that the increased binding of Na^+ to brain
lipoprotein was not due to ATP specifically acting as a
substrate, but rather due to the nucleotide chelating Mg^{++},

and leaving more sites available to bind Na^+. This same

effect could be demonstrated using EDTA, but not EGTA, a

poor Mg^{++} chelator. This work showed that the binding of

various ions to rat brain lipoprotein is quite non-specific,

since Na^+, K^+, Li^+ and Mg^{++} all bind well.

As a result of this work, any conclusions regarding

specific binding of ions to a phosphorylated intermediate

should be critically analyzed.

In 1963, Glynn reported the existence of an ATPase

system in the electric organ of Electrophorus electricus,

which possessed the usual cationic characteristics.

That same year, Albers and co-workers (1963) pre-

sented material indicating the presence of a phosphoryl-

ated intermediate in this tissue, and postulated the

following mechanism which has since been substantiated

(Albers et al, 1966; Fahn et al, 1966).

$$ATP + XEY \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} ADP \quad P \sim XEY$$

$$P \sim XEY \underset{}{\overset{Na^+}{\rightleftharpoons}} XEY \sim P$$

$$XEY \sim P + H_2O \underset{}{\overset{K^+}{\rightleftharpoons}} XEY + P$$
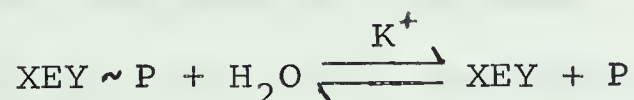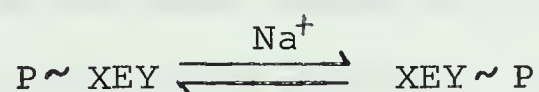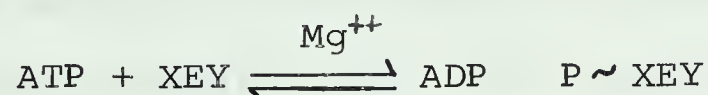
The newly postulated component was the Na^+ stimulated

transferase which alters the structure of the phosphoryl-

ated intermediate compound to allow the K^+ stimulated hydro-
lysis to occur.

Ahmed and Judah (1965), Hokin et al (1965) and Gibbs
et al (1965) have demonstrated the existence of a phosphory-
lated intermediate compound in brain tissue. Hokin has
shown that his intermediate is more stable to alkali than
acid, and that digestion of the protein with pepsin after
previous P^{32} incubation releases two major radioactive
peptides. Treatment of these peptides with hydroxylamine
acetate or with acyl phosphatase prepared from the trans-
port ATPase liberated most of the radioactivity from these
peptides as P_i^{32}. This observation led these workers to
suggest that one phosphorylated intermediate in the trans-
port ATPase is probably an acyl phosphate.

Bader and Sen (1966) and Bader et al (1966) showed a
close relationship between the rates of hydrolysis of acetyl
phosphate and ATP by the transport ATPase of guinea pig
kidney cortex preparations, with respect to activation by
cations, pH optimum, sensitivity to inhibitors, and treat-
ment with Triton and acetone. Each substrate also was a
competitive inhibitor of the other. This close relation-
ship suggested that the K^+ dependent acetyl phosphatase is
the same as the K^+ dependent hydrolysis of the phosphoryl-
ated intermediate of the Na^+-K^+ dependent ATPase, thus imply-
ing that the phosphorylated intermediate is an acyl phos-
phate.

However, Schoner et al (1966) tested this possibility by investigating the effect of hydroxylamine (H_2NOH) on the overall hydrolysis of ATP in ox brain microsomes. Their hypothesis was that hydroxylamine, by combining with the intermediate should irreversibly inactivate the ATPase.

$$1)\ ATP + E \xrightleftharpoons{Mg^{++}, Na^{+}} E \sim P + ADP$$

overall hydrolysis

$$2)\ E \sim P + H_2O \xrightarrow{K^{+}} E + P$$

$$3)\ ECOOH + ATP \xrightleftharpoons{Mg^{++}, Na^{+}} E - \overset{O}{\overset{\|}{C}} OP + ADP$$

labelling

$$4)\ E\overset{O}{\overset{\|}{C}}-OP + H_2O \xrightarrow{K^{+}} ECOOH + P_i$$

$$5)\ E\overset{O}{\overset{\|}{C}}-OP + NH_2OH \rightarrow E\overset{O}{\overset{\|}{C}}NHOH + P_i$$

H_2NOH effects

$$6)\ E\overset{O}{\overset{\|}{C}}NHOH + H_2O \rightarrow ECOOH + NH_2OH$$
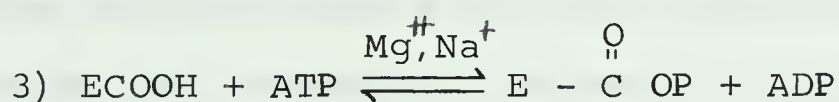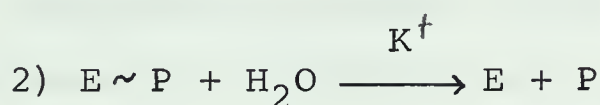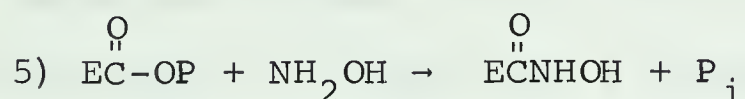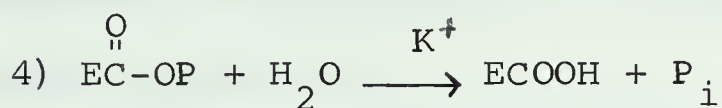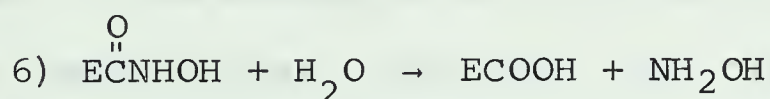
If the E bound H_2NOH were unstable (step 6) H_2NOH should substitute for K^{+} in the overall reaction. None of these postulates agreed with the experimental data. Na^{+} and K^{+} stimulated ATP hydrolysis was not inhibited even at very high concentrations of H_2NOH. Also in the presence of this chemical optimal rates of ATP hydrolysis depended upon the presence of K^{+} ions. As a result of

these observations, these workers excluded a role of the
enzyme bound acyl phosphate in the overall reaction. They
further demonstrated that both ADP and IDP can decrease
the labelling of the intermediate compound, yet only ADP
can act as a competitive inhibitor of the overall reaction
(hydrolysis of ATP). They therefore conclude that the
participation of a phosphorylated intermediate is doubtful.

Judah and co-worker (1962) have shown that red blood
cells contain a phosphoprotein with properties similar to
the ones mentioned above by Hokin, Post and others. In
this case, Na^+ internally increased the labelling of the
intermediate, while external K^+ caused a breakdown, which
was ouabain sensitive.

Heinz and Hoffman (1965), although finding results
which led them to include such a phosphorylated inter-
mediate in the mechanism of ATP hydrolysis in red blood
cells, nevertheless questioned the actual role of the
intermediate. They divided the total P^{32} content of red
blood cell ghosts into a perchloric acid (PCA) soluble
fraction, and a PCA insoluble fraction. The PCA soluble
fraction was composed primarily of ATP^{32} and some P_i^{32}.
The PCA insoluble fraction contained only P_i^{32}. If the
P^{32} in this latter fraction represented an intermediate
in membrane ATPase activity, it should have been exchange-
able on restitution of the ATPase activity. This P^{32} was

found to be stable and nonexchangeable upon the reincubation of prelabelled ghosts under conditions which reactivated the ATPase. An actual mechanistic explanation was not offered, and the only suggestion made was that the results were artefacts. Either the intermediate was masked by nonspecific P^{32} binding, or it resided in the PCA soluble fraction.

In summary, it can be said that at the moment there is considerable debate as to the actual mechanism of the ATPase activity. The evidence, although not overwhelming, seems to point to a phosphorylated intermediate involved in the reaction as follows:

$$ATP + x \quad \overset{Na^+}{\rightarrow} \quad X \sim P_i + ADP$$

$$x \sim P_i \quad \overset{K^+}{\underset{H_2O}{\rightarrow}} \quad X + P_i$$
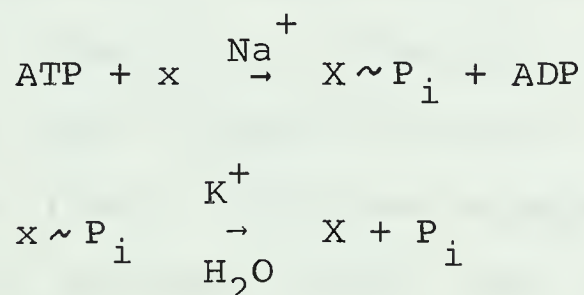
It should be noted that no one group of workers has taken one tissue and performed all these experiments mentioned above. In order for one to draw conclusions, one must interpolate from tissue to tissue. It is hoped that one tissue will be examined from all these approaches in the very near future.

B. <u>Chemical Compounds and Various Treatments that</u> <u>Alter the Activity Ratio</u>

In discussing the transport ATPase, one can readily refer to the activity ratio. This is defined as the activity of the enzyme preparation in the presence of Mg^{++}, Na^+ and K^+, divided by the activity of the enzyme in the presence of just Mg^{++} ($Mg^{++} + Na^+ + K^+/Mg^{++}$). Since this enzyme is stimulated by the addition of Na^+ and K^+, the activity ratio will always be unity or greater than unity. As this section progresses, it will be seen that the question of one or two enzymes comprising the activity is an integral part of the above heading, and that the mechanism of any treatment, chemical or otherwise, cannot be fully explained until the above dilemma is solved.

Skou (1963, 1965a) and others (for a review of all literature, see Skou, 1964, 1965) have demonstrated that the Na^+, K^+ stimulated ATPase is inhibited by a number of sulphydryl inhibitors, including PCMB, NEM and DNFB. The inhibition by PCMB could be reversed by cysteine. The activity ratio was increased by both NEM and DNFB, although both the $Mg^{++} + Na^+ + K^+$ and the Mg^{++} activities decreased, the latter much faster than the former, and thus increasing the activity ratio. ATP partially (Skou and Hilberg, 1965) protected the system against the inhibitory effect on the $Mg^{++} + Na^+ + K^+$ activity, thus further increasing the

activity ratio. ADP, but not ITP, had the same protective effect as ATP.

Urea had the same effects as NEM and DFNB, and its effects on the enzyme were protected against by ATP, again increasing the activity ratio.

These results led Skou to postulate that the effects of NEM and DFNB are not primarily due to their action as sulphydryl group inhibitors, but rather due to a change in the steric configuration of the enzyme. This is necessary, because it seems difficult to explain the protective effect of ATP by combination of the nucleotide with sulphydryl groups. Skou further postulated that one enzyme alone could be responsible for both the Mg^{++} and the $Mg^{++} + Na^+ + K^+$ activities.

This last question is by no means settled. Nakao and co-workers (1963) have isolated two separate enzyme activities from red blood cell membranes. Most other workers try to increase the activity ratio without isolating the Mg^{++} stimulated enzyme. This former group, by the use of NaI treatment, actually isolated two separate enzyme activities, one being Na^+, K^+ and ouabain sensitive in the usual manner, and the other being relatively insensitive to all the above. At least in this tissue, one can be sure that there is no contamination with mitochondrial ATPase.

Samaha and Gergely (1965, 1966) have shown that the

microsomal fraction of human striated muscle contains a MgATPase activity which is insensitive to ouabain and inhibited by very low concentrations of NaN_3, oligomycin, and quinidine. This fraction also contained the usual Na^+ and K^+ activated ATPase, which was completely inhibited by ouabain, and whose activity was enhanced by the presence of the above three compounds. (The activity ratio was increased since these compounds lowered the Mg^{++} activity.)

These workers further demonstrated that similar concentrations of these three agents caused similar degrees of inhibition of the MgATPase activity of the mitochondrial fraction, and that the mitochondrial ATPase was inhibited by Na^+ and K^+. These experiments, and experiments with mixtures of aged microsomal fractions, and fresh mitochondrial fractions (which together resembled fresh microsomal fractions) suggested that the fresh microsomal fraction contained two ATPases, one present in mitochondrial fragments, and the other present in the microsomal membranes. The Na^+ inhibition of the former, unless eliminated by ageing or by mitochondrial ATPase inhibitors masks the activity of the $Na^+ + K^+$ ATPase. Fratantoni and Askari (1965) were unable to demonstrate $Na^+ + K^+$ stimulation of a MgATPase from skeletal muscle.

Auditore and Wade (1964) have demonstrated the presence of Na^+ and K^+ activated ATPase in rabbit kidney mitochondria, and have also shown that the presence of desoxycholate in the isolating medium causes extensive mitochondrial disruption, and the production of many small fragments which would easily contaminate the higher speed sediments. Since desoxycholate is often employed in isolation of membrane fragments, this could conceivably cause mitochondrial contamination. By the same measure, it is possible that the kidney mitochondria could be contaminated with fragments of microsomal material (see Charnock and Post, 1963a,b, and Landon and Norris, 1963). Schwartz (1964) has shown $Na^+ + K^+ + Mg^{++}$ stimulation of liver microsomal ATPase.

To date few studies have used both extensive electron microscopy and biochemical techniques to determine the contamination of various fractions, and as a result, the question of one or two enzymes being responsible for the Mg^{++} and $Mg^{++} + Na^+ + K^+$ activities remains unanswered.

Schwartz (1962, 1965) and Schwartz and Laseter (1964 a,b) have shown with cardiac muscle that ageing of the microsomal preparation increases the activity ratio. They also found that the basic protein histone could inhibit the Mg^{++} stimulated ATPase activity more than it could the $Na^+ + K^+ + Mg^{++}$ ATPase activity, and thus could increase the activity ratio. Ageing, therefore, might allow nuclear

histones, which may not have been released during homogen-
ization, to interact with the enzyme. The ageing procedure
gives best results when carried out in the cold. These re-
sults may have significant implications for cellular regu-
lation in general, since it is conceivable that these nuc-
lear proteins may regulate enzyme production.

This result with histone, and Schwartz's earlier work
showing the presence of a soluble heat stable factor in
both parotid gland and muscle which when added to a micro-
somal preparation increased the activity ratio (Schwartz and
Laseter, 1964), have led Skou (1965) to suggest that some-
thing is removed from the enzyme during preparation, and
that the removal changes the enzyme from a Mg^{++} + Na^+ + K^+
sensitive one to only a Mg^{++} sensitive one.

Thus Skou belives in the presence of only one enzyme,
and that the Mg^{++} activity, insensitive to Na^+ and K^+, is
an artefact of isolation.

With regard to Schwartz's result with histone, it
is interesting to note that Yoshida et al (1965) found that
pretreatment of a brain ATPase microsomal preparation with
protamine at both 37^OC and 45^OC resulted in a loss of
Na^+ + K^+ stimulated ATPase activity, but had no effect on
the Mg^{++} stimulated ATPase, thus indicating the histone
effect is specific and not merely the result of a posi-
tively charged macromolecule, i.e. the effect does not

appear to be simply a consequence of electrostatic charge cancellation.

Much other work has been done with chemicals as inhibitors, but these results cannot be placed in context at present. These results have been included briefly in the chart on page 46.

C. Mechanism of Ouabain Inhibition

At present the mechanism of action of ouabain inhibition is unclear (Glynn, 1964), although many workers have shown that the effect of the glycoside may be overcome by increasing the K^+ concentrations. Whittam (1958) showed in reconstituted red blood cell ghosts that ouabain inhibited Na^+ and K^+ transport and ATPase activity only if left on the outside of the cell, presumably in contact with the external membrane surface. If the glycoside was incorporated inside the cell by reversible hemolysis, it had no effect. The inhibitory effects on both transport and enzymic activity were overcome by increased external K^+.

Caldwell and Keynes (1959) have shown that ouabain demonstrates similar effects on Na^+ transport in nerve fibers.

Repke et al (1965) showed very large species variation when testing the inhibitory effect of ouabain, digitoxin, cassaine, and prednisoline-3,20-bis-guanylhydrazone.

These workers speculated that complementarity between the surface of the steroid or terpenoid component of the inhibitor, and the surface in the neighborhood of the K^+ center of the ATPase is the decisive factor for the inhibitory activity of the compounds, indicating competitive inhibition.

Charnock et al (1963a,b) demonstrated that Na^+ was necessary for the formation of a phosphorylated intermediate, and K^+ induced its hydrolysis. Ouabain, however, was able to overcome the hydrolytic effect of K^+.

Further studies by Post et al (1965) showed that ouabain was able to partially inhibit the Na induced formation of the P^{32} labelled intermediate, as well as the K^+ induced hydrolysis. Ouabain could increase the amount of the intermediate as it decreased the overall rate of ATP hydrolysis at low K^+ concentrations, but at high K^+ concentrations, the amount of intermediate and rate of hydrolysis were lowered together. Furthermore, in the absence of K^+, ouabain inhibited the formation of the phosphorylated intermediate. It was suggested that ouabain could inhibit both inward and outward translocation of the intermediate. Translocation in this paper refers specifically to the step involved in the transport reaction which allows the active site of the system to change the side of the membrane with which it is in contact. In the postulated

scheme the enzyme is translocated from the inside of the cell (E_i) to the outside (E_o) with Na^+ attached and from outside to inside with K^+.

$$
\begin{array}{l}
P_i \\
+ \\
2K^+ \\
+ \\
E_i + 3Na^+ + ATP \rightleftharpoons ADP + Na_3E_i\text{-}P \\
\end{array}
\qquad \text{IN}
$$

$$
K_2E_o\cdot P + 3Na^+ \longleftarrow 2K^+ + Na_3E_i\text{-}P \qquad \text{OUT}
$$
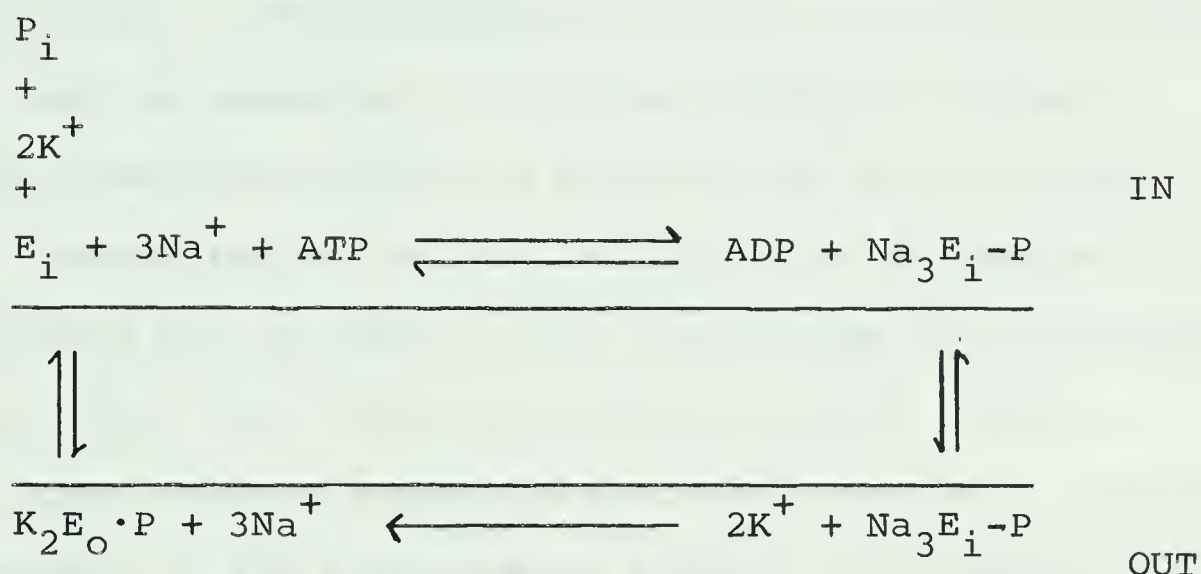
Thus ouabain inhibition of both inward and outward trans-location could cause an equal accumulation of E_i and E_o, and phosphorylation of E_i and dephosphorylation of E_o would go to completion. The amount of P^{32} intermediate would only be half maximal.

Recently, Matsui and Schwartz (1966) have concluded from a kinetic analysis of the enzyme from cardiac tissue, using both Lineweaver-Burk and Dixon plots, that inhibition by ouabain is due neither to direct nor indirect displace-ment of K^+ at the K^+ site. They further concluded that there was a dependency of the K_i for ouabain on the Na^+/K^+ ratio, and that the ouabain and K^+ sites were not the same.

Palmer et al (1964, 1966) have shown that at low concentrations (10^{-12} - 10^{-9} M) ouabain can stimulate the ATPase activity of both chicken kidney and rabbit brain microsomes. Na^+ and K^+ must be present for this stimulation to occur. This stimulatory effect has characteristics which tend to separate it from the inhibitory effect: a) the stimulatory effect is not altered by alterations in the K^+ concentration, while inhibition is; b) ageing of the preparation for 14 days at $-20^{\circ}C$ diminishes the stimulatory effect, while the inhibitory effect is still present.

These workers suggested that there may be 2 receptors for ouabain in the preparations studied; both Na^+ and K^+ dependent, one eliciting stimulation, and the other inhibition of ATP cleavage.

D. Phospholipid Requirements

Interesting results were obtained by Tanaka and his associates (1964, 1965) concerning the phospholipid requirement of brain microsomal ATPase. They found that solubilization of the enzyme with desoxycholate resulted in a loss of Na^+ and K^+ activity which could be restored by the addition of various phospholipids, and that the loss of enzyme activity paralleled the loss of phospholipids.

It was also observed that only in the presence of phospholipid was the ATPase activated by Na^+ and K^+, and

inhibited by ouabain. Lecithin also protected the enzyme against the effects of the sulphydryl inhibitor, NEM, as well as against heat inactivation, but did not modify the effect of ouabain, or Na^+ plus K^+. The phospholipid did not affect the ATP-ADP^{32} exchange reaction, or the labelling of the phosphoprotein. Further studies indicated that the preparation contained at least two different types of ATPases; one associated with Na^+ and K^+ stimulation, requiring phospholipid, and one not associated with Na^+ and K^+ stimulation, and lacking the requirement.

Emmelot and Bos (1965, 1966a,b) isolated a membrane preparation that was not a microsomal fraction. They found that removal of the terminal o-glycosidic linked sialic acid from a membrane glycoprotein, by the action of neuraminidase affected both the Mg^{++} stimulated ATPase (inhibition) and the $Na^+ + K^+$ stimulated ATPase activity (activation). The latter effect depended upon the basic control enzyme activity. If the controls were of low or medium activity, the neuraminidase treatment would cause activation. If the control activity was high, the enzyme treatment would inhibit the Na^+ K^+ ATPase activity.

Despite the large amount of work that has been done on this enzyme system, no concrete mechanism has been worked out. Although many different types of tissues have been studied, including skeletal and cardiac muscle, no

work has been published on the $Na^+ + K^+ + Mg^{++}$ ATPase activity of smooth muscle.

This fact, coupled with the observed differences of ion fluxes resulting from the active transport inhibition in rat uterus as mentioned earlier, indicated that a study of the ATPases of this tissue would be most interesting.

VARIOUS ATPases AND THEIR PROPERTIES

ATPase	Substrates	Cofactors	Activators and Stimulators	Inhibitors
Myosin (E.C. 3.6.1.3)	ATP>CTP>UTP >ITP>GTP Triphosphate Many synthetic analogues	Ca^{++}	DNP	Mg^{++}, Cu^{++}, Ag^{++}, PCMB, $1MK^{+}$, NEM
Actomyosin (E.C. 3.6.1.4)	"	Ca^{++}, Mg^{++}, in presence of actin		All above, except Mg^{++} A-S' sulfato-pyrophosphate
Endoplasmic Reticulum (E.C. 3.6.1.4)	ATP>ITP<hr>Microsomal ITP>GTP>CTP ATP>UTP	Mg^{++}	Ca^{++} (extra ATPase splitting)	Ca^{++} Not by $1MK^{+}$
Mitochondria Intact (E.C. 3.6.1.4)	ATP>ITP>GTP >CTP>UTP	For ATP Mg>Mn>Fe> Co>Ca	DNP (only for ATP) In absence of Mg^{++}	Na^{+}, K^{+}, NaN_3, PCMB, guanidine, thyroxin analogues, bilirubin, ADP, oligomycin (ATP only)
Mitochondria Soluble	ITP>ATP>GTP >CTP>UTP	Mg^{++}>Co^{++}> Ca^{++}>Fe^{++}	DNP (only for ATP)	" Not by oligomycin. Cold labile
Membrane (E.C. 3.6.1.4)	ATP>ITP> GTP>UTP	Mg^{++}	Na^{+}, K^{+} (only for ATP)	Ca^{++}, ouabain heavy metals F^{-}, PCMB oligomycin

(E.C. = Enzyme Commission Classification)

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METHODS AND MATERIALS

I. <u>Total Homogenate</u>

A. <u>Preparation</u>

Female Wistar rats weighing 175-225 gms., were pre-treated with 100 γ diethylstilbestrol per day sub-cutaneously for 6 days and were killed by a blow on the head. This amount of hormone was used because preliminary studies of the total homogenate ATPase activity showed it produced maximal enzyme activity. The peritoneal cavity was opened, the uterine horns excised, and the endometrium and circular muscle layer separated from the longitudinal layer. This was accomplished by splitting the horn lengthwise, laying it flat on a piece of filter paper, and peeling off the endo-metrial and circular muscle layers. The resulting longitu-dinal muscle from each horn usually weighed about 50-100 mg. Three or four rats were usually used for each experiment. The longitudinal muscle was then placed in cold distilled and demineralized water (Crystalab Deeminizer®) and homo-genized in a Potter-Elvejhem ground glass homogenizer, to achieve a suspension of 10% (w/v). All steps were carried out in the cold (0-4°C).

B. <u>Assay</u>

Various assay media were prepared. The basic (com-plete) assay medium contain 1 mM $MgCl_2$, 58 mM NaCl, 5 mM KCl, 10 mM NaCN, .1 mM EDTA, 92 mM Tris

(Bonting, 1961). The substrate was added prior to the addi-
tion of the homogenate. The Tris salt of ATP (Sigma Bio-
chemical Co.) was used in a concentration of 2 mM. Other
nucleotides were used in similar concentrations. With ATP
as substrate, alterations of the Mg/ATP ratio did not alter
the enzyme activity. When other assay media were made which
contained additional compounds, appropriate changes were
made in the Tris concentration to maintain isotonicity.
The low Na^+ medium contained 5 mM NaCN and the K^+ level
was maintained with 5 mM KCN. The medium with no ions
actually contained 5 mM KCN and 5 mM NaCN, (CN^- inhibits alka-
line phosphatase). The final incubation mixture contained
twice as much assay medium as homogenate, usually 3 ml.
and 1.5 ml., or 4 ml. and 2 ml.

The assay medium was prewarmed in a Labline shaker-
water bath (Labline Instruments Co., Melrose Park, Ill.)
at 37°C after the substrate had been added. All incuba-
tions were carried out at 37°C with shaking, in this instru-
ment.

The various substrates were added in the following
manner. The absolute amount of substrate to be added to
each assay tube was determined. This value for each tube,
and the number of assays to be run, gave the total amount
of substrate used for the experiment. This total was
weighed out, and dissolved in a quantity of distilled and

demineralized water, so that this absolute amount of sub-
strate per tube would be contained in a .2 cc. volume

This amount was then added to the assay medium, and
the pre-warming begun. No appreciable amount of substrate
hydrolysis could be detected under these conditions, when
carried out for as long as 10 minutes. The pre-warming
usually lasted about 2 minutes.

The tissue homogenate was also pre-warmed, and after
2 minutes, was added to the assay medium, initiating the
reaction. The reaction was stopped by withdrawing a 1 cc.
aliquot of the reaction mixture, and placing it into 2 cc.
of a 15% (w/v) solution of TCA. The mixture was then
spun in a clinical centrifuge at maximum speed for 5 min-
utes, and the supernatant retained for the determination
of inorganic phosphate by the Fiske-Subbarow method.

II. Cellular Fractionation

    A. Preparation

    Rats were killed and uteri excised and dissected
as previously described. The longitudinal muscle layers
were placed in the appropriate isolation medium, usually
.25 M sucrose, weighed, and then homogenized in a Potter-
Elvehjem ground glass homogenizer, or in a VirTis "23"
homogenizer (The VirTis Co. Inc., Gardener, N.Y.). The

various isolation media used will be discussed in the results section.

It was shown that the ground glass type homogenizer released free Na^+ (about .2 mM) into the medium, so the blade type VirTis was also tried. As far as the methods could determine, the presence of that amount of Na^+ gave no different results in the following types of experiments: Na^+, K^+ stimulation, in both stored and fresh fractions; Ca^+ and Mg^{++} interaction; ouabain and NEM inhibition; basic Mg ATPase and Ca ATPase activities.

The homogenate was then centrifuged in a clinical centrifuge (International, Model CL) at approximately 600 x g, the supernatant saved, the sediment resuspended, centrifuged against the same force, and the two supernatants combined. The sediment was called the nuclear fraction and was assumed to contain nuclei and all debris.

The combined supernatant was then spun at 18,500 x g (13,500 rpm) for 15 minutes in a Spinco Model L ultracentrifuge. The supernatant was saved, the pellet resuspended, and centrifuged again. The supernatants were combined, and the pellet was designated the mitochondrial fraction. In some experiments, a centrifugal force of 36,000 x g (20,000 rpm) for 15 minutes was used.

The combined supernatants were then spun at 127,000 x g (37,500 rpm) or 115,000 x g (36,800 rpm) for 60 minutes

to obtain a microsomal fraction and final supernatant.

The three fractions, nuclear, mitochondrial and micro-
somal, were resuspended in various media for testing. The
volume of suspending buffer used was either 4 cc., 8 cc.,
or 10 times the number of grams of original tissue used.
These speeds were those used by Wakid (1960).

B. Assay

The assay procedure for the subcellular fractions
differed from that described for the total homogenates.
The basal media contained an appropriate buffer, various
concentrations of $MgCl_2$ and substrate. The amount of
substrate to be used was added as previously described
for the total homogenate. Other ions or chemicals were
usually added to the medium dissolved in buffer or double
distilled water in as small a volume as possible. In all
cases of assays of the enzymatic activities of the frac-
tions, the volume of the assay media was kept to 3 cc.
or 6 cc., depending upon the number of aliquots to be with-
drawn.

The 1 cc. aliquots were treated as described above
when P_i was to be determined by the Fiske-Subbarow method,
and placed directly into the proper reagent when the
LeCocq-Inesi method was to be used.

The hydrolysis of ATP (Tris salt was always used)
by the microsomal fraction was linear up to 6 minutes, and

FIGURE 1.
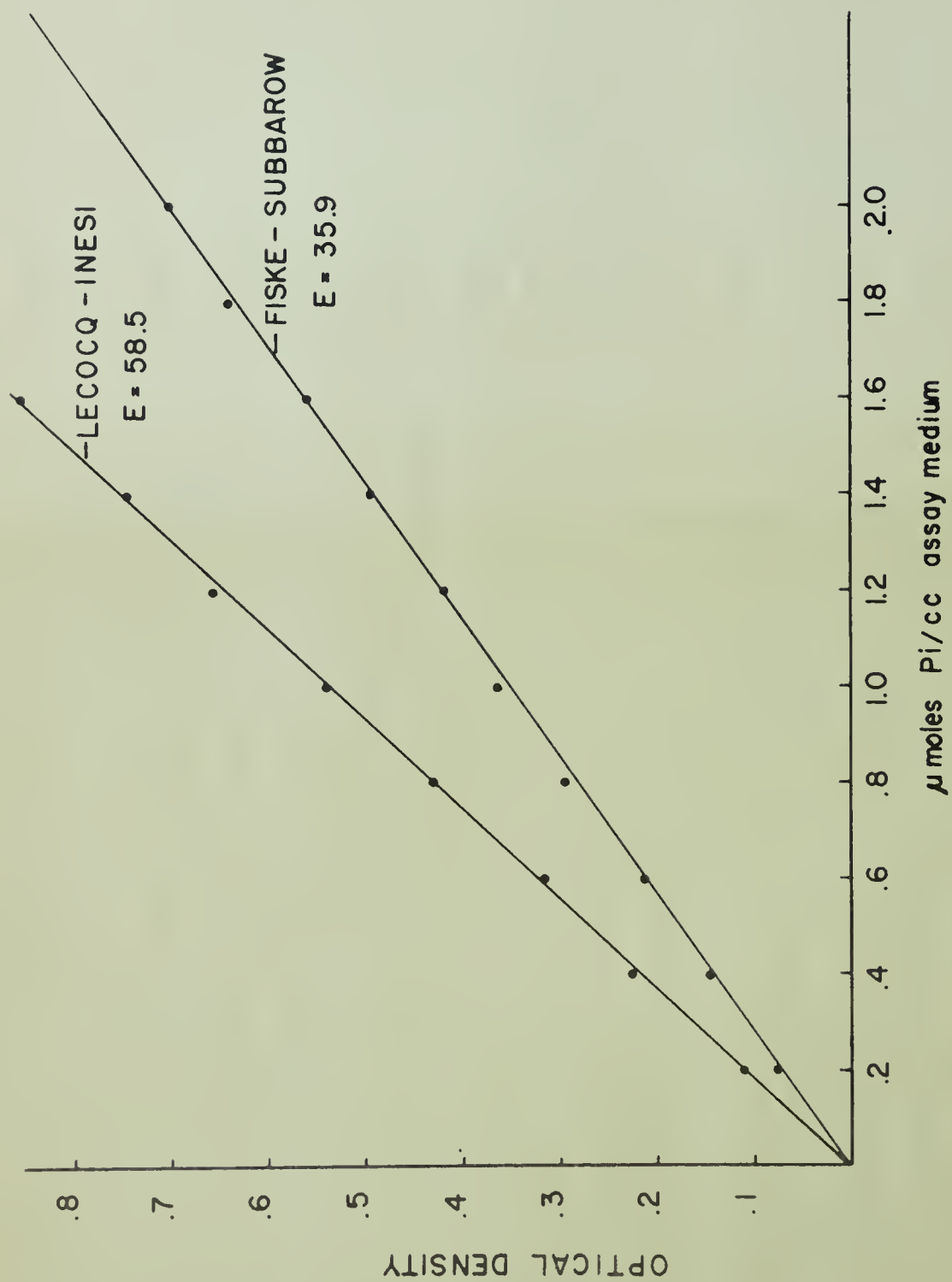
on extrapolation passed through the origin, thus justify-
ing aliquots being taken at 5 minutes. At this 5 minute
interval, less than 20% of the substrate normally was
hydrolyzed to ADP.

III. Determination of Inorganic Phosphate (P_i)

The Fiske-Subbarow method was used for most of the
work described. Later the LeCocq-Inesi method was des-
cribed in the literature and was then used in preference
to the Fiske-Subbarow, for the following reasons:

1) It is a one-step method, in contrast to the
Fiske-Subbarow, a 3-step method.

2) The sensitivity of the LeCocq-Inesi is higher
(ε = 58.5) than the Fiske-Subbarow (ε = 35.9).
(ε is the specific extinction coefficient, and
may be defined as the extinction or optical
density per unit thickness (light path length
through the solution) per unit concentration)

3) The color formed by the LeCocq-Inesi method is
stable for at least 30 minutes, while the Fiske-
Subbarow color complex is stable for only 10
minutes.

The LeCocq-Inesi method is thus easier, more reli-
able and more sensitive.

1) Fiske-Subbarow Method (FS)

Reducing agent

Mix thoroughly,

   .2 g 1-amino-2-naphthol-4-sulfonic acid

   1.2 g $NaHSO_4$

   1.2 g Na_2SO_4

Use a mortar and pestle to grind to a fine powder.

Store the reducing agent in a dark bottle, away from

light. For use, dissolve .25 g in 10 cc. double dis-

tilled water.

Steps:

(1) A 1 cc. aliquot from the assay medium is added to

   2 cc. cold 15% TCA.

(2) The mixture is centrifuged at maximum speed in a

   clinical centrifuge and the supernatant saved for

   P_i determination.

To the 3 cc. supernatant, add

   1 ml. 5 N H_2SO_4

   1 ml. 2.5% $(NH_4)_6MO_7O_{24}$

   .1 ml. reducing agent

Then bring up to 10 cc. with double distilled water.

After 10 minutes, read on a Beckman DU spectrophoto-

meter at 660 mμ, slit width .015, phototube load

resistor on 1.

2) LeCocq-Inesi method (LI)

Make up the following reagents.

(1) $(NH_4)_6 \cdot MO_7O_{24} \cdot 6H_2O$ 100 g

     NH_4OH (specific gravity .9)   10 ml

     Make a 1 liter solution with double distilled water.

(2) Add 2.35 g. NH_4VO_3 to 400 cc. of hot double distilled water, and cool under the tap when dissolved. Add 6.16 cc. HNO_3 (specific gravity 1.42) previously diluted with 14 cc. double distilled water, and bring to 1 liter with double distilled water.

(3) Add 150.5 g. to 100 cc. reagent 1, and 100 cc. reagent 2, and bring to 500 cc. with double distilled water.

Steps:

(1) Add a 1 cc. aliquot from the assay medium to reagent 3.

(2) Centrifuge at maximum speed in a clinical centrifuge, and save the supernatant.

(3) One cc. of this supernatant is then brought to 10 cc. with double distilled water, and read at 350 λ on a Beckman DU spectrophotometer (slit width, .04; H_2 lamp, 3; screen bias, 5; sensitivity, 4; zero supplement off).

IV. Method for Calculating Enzyme Activity

A. Total Homogenate

A 10% (w/v) homogenate was prepared, so that there was 100 mg. tissue per ml. suspension.

For example, 4.5 ml. of final medium composed of 3.0 ml. assay medium and 1.5 ml. tissue suspension contained a total of 150 mg. tissue. If a 1ml. aliquot of this mixture after incubation gave an optical density reading of .490, corresponding to 5 μmole P_i, from the standard curve, the total assay medium would then contain (4.5)(5) = 22.5 μmole P_i/4.5 ml., or 22.5 μmole P_i/150 mg. tissue, or (22.5)(6.7) = 148.5 μmoles P_i/g. wet wt. of tissue/unit time.

B. Subcellular Fractions

Example

Kjeldahl analysis showed that the microsomal fraction contained an average of 1.20 mg N for each gram wet weight original tissue (see results section, Table III). Thus .5 g. tissue contained approximately .600 mg. N which was subsequently isolated in the microsomal fraction.

If such a pellet was homogenized in 5 ml. of buffer, the suspension contained .600 mg N per 5 cc., or .12 mg N per cc. If .2 cc. of this microsomal suspension was used for the 5 minute assay, then the assay medium contained .024 mg. N.

Assume that the spectrophotometric reading and standard curve gave a value of .67 μmoles P_i/1 cc. aliquot, or 2.0 μmoles P_i/3 cc. assay medium/5'. This is equal to 2.0 μmoles P_i/.024 mg N/5', or 83.3 μmoles P_i/mg N/5'.

In general, the following formula was used to calculate enzyme activity.

The amount of P_i liberated/mg N/unit time =

$$\left(\begin{array}{c} \text{volume of} \\ \text{assay medium} \end{array} \right) \left(\begin{array}{c} \text{total volume} \\ \underline{\text{of suspension}} \\ \text{vol. of sus-} \\ \text{pension used} \\ \text{for assay} \end{array} \right) (F_x) \left(\begin{array}{c} \mu\text{moles } P_i/\text{cc.} \\ \underline{\text{assay medium}} \\ \text{unit time} \end{array} \right)$$

where $F_x = \dfrac{1}{(\text{gm. tissue wet wt.}) \dfrac{\text{mg. N in the fraction}}{\text{gm. wet wt. of tissue}}}$

The volume of suspension in cc. was usually 10 times the number of grams in the original wet weight of the tissue, although in some experiments the volume was 4 or 8 cc.

The above formula could be used for any subcellular fraction, knowing the amount of nitrogen contained in that fraction (see results), and the wet weight of the tissue (i.e. F_x).

V. Cell Membrane Isolation

The isolation of cellular membranes of this tissue was based on the procedure of Rosenthal et al (1965) as modified by Carroll (to be published).

Solutions

1) KCl buffer

The following stock solutions were made up:

450 mM KCl

300 mM $KHCO_3$

25 mM D-histidine HCl

1 M Tris

Four drops of the Tris are added to 5 ml. of each of
the first 3 solutions. This mixture is then brought
to 50 cc. with distilled, demineralized water. The
final concentrations in the buffer are:

45 mM KCl

30 mM $KHCO_3$

2.5 mM histidine HCl

The buffer also contains small amounts of Tris, added
to bring the pH to 7.8. The effect of the presence of
both histidine and Tris is unknown, because of results
obtained with these compounds in the microsomal frac-
tion (see results).

2) NaOH

One pellet was dissolved in 50 cc. Twelve μl. of this
solution was diluted to 500 cc. This gives a NaOH con-
centration of about $2-3 \times 10^{-7}$ N.

The uteri were excised, and the endometrium stripped off in the usual manner.

1) Glycerol Extraction

The tissue was placed in the following glycerol-Krebs mixtures:

1:9 glycerol-Krebs (w/v) $37^{O}C$ 2 hrs

2:8   " $0^{O}C$ 1/2 hr

3:7   " $-4^{O}C$ 1/2 hr

To make the glycerol-Krebs solution, the appropriate amount of glycerol (1, 2 or 3 grams) was weighed in a previously tared 10 cc. volumetric flask. The solution was then brought to 10 cc. with the Krebs medium. After the first incubation, the tissue was then frozen on a block of CO_2, cut with a scalpel, and set into blocks with 50 mM $CaCl_2$. These blocks were then placed in foil, and stored in liquid nitrogen for varying lengths of time.

2) Tissue Sectioning

The blocks of tissue were mounted on a Leitz-Wetzlar freezing microtome, and sectioned at 5 μ. The resulting mush-like material was placed in cold 50 mM $CaCl_2$.

3) Centrifugation and NaOH Treatment

All centrifuging was done in a clinical centrifuge at

maximum speed.

a) The above material was spun and the supernatant
    decanted.

b) The pellet was resuspended in KCl buffer and cen-
    trifuged twice. Each time the supernatant was
    discarded. The volume of buffer used varied be-
    tween 5 and 10 cc.

c) The pellet was resuspended in buffer, and incubated
    for 1/2 hr at 37°C and then placed in ice.

d) The suspension was centrifuged 3 times, and each
    time the pellet was resuspended in buffer, and
    allowed to settle in ice water before centrifuation.

e) The above procedure was repeated using double dis-
    tilled water instead of buffer.

f) The above procedure was repeated 5-6 times using
    NaOH instead of water. The volume used was still
    not critical, and between 5 and 10 cc. was used.

g) The residue was suspended in 10 cc. of NaOH, and 1
    ml of a 2 mM ATP was then added, mixed gently and
    the total added to 75-100 ml double distilled water.

h) The suspension was rinsed with double distilled
    water 2 or 3 times, each time centrifuging and dis-
    carding the supernatant.

i) The pellet was resuspended in double distilled water
    for storage.

VI. <u>Nitrogen Determination of Subcellular Fractions by</u>
Micro Kjeldahl Method

These determinations were done on three separate
occasions. Each time the procedure was the same.

Twelve rats were killed as previously described
and the combined uteri were homogenized in 15 cc. of .25
M sucrose, using only a ground glass homogenizer. Three
cc. of this homogenate was used for nitrogen determina-
tion of the total homogenate. (1 cc. per determination).

The remaining 12 cc. were centrifuged as usual, and
each resulting fraction was suspended in 6 cc. of .25 M
sucrose.

Each of these 4 suspensions was then divided in
half, and the nitrogen in each half determined.

Knowing the original tissue weight, the number of
mg. N/gm. wet weight of tissue could be calculated for the
whole tissue, and for each subcellular fraction.

Micro Kjeldahl Determination of Protein Nitrogen

I. Reagents:
  1. Methyl Red - Methylene Blue Indicator.
     Mix 2 parts of 0.2% methyl red with 1 part methyl-
     ene blue (0.2%) solution, both in alcohol.
  2. Sodium Hydroxide - Sodium Thiosulfate solution.
     Dissolve 50 gm. NaOH and 5 gm. $Na_2S_2O_3 5H_2O$ in water

and dilute to 100 ml.

Where large quantities are being used it will be found convenient to use the following mixture:

670 gm. NaOH

67 gm. $Na_2S_2O_3 5H_2O$

1350 ml. water

3. Boric Acid Solution.

Dissolve 4 gm. H_3BO_3 in 100 ml. H_2O. This solution can be made up in large quantities.

4. Mercuric Sulfate (10%).

Dilute 12 ml. concentrated H_2SO_4 to 100 ml. and add 10 gm. of HgO, stirring until the solid dissolves.

5. Reagent K_2SO_4.

6. Reagent concentrated H_2SO_4.

7. 30% hydrogen peroxide.

8. Demineralized water.

II. Procedure:

(a) Digestion

1. Place in each flask approximately 0.50 gm. K_2SO_4.

2. Add 1 ml. of the solution to be analysed using a 1 ml. volumetric pipette.

3. Add 0.5 ± 0.05 ml. of $HgSO_4$ solution.

4. Add 3.0 ± 0.1 ml. of concentrated H_2SO_4.

5. Digest until clear.

6. Remove flasks from the heat and allow to cool for 3-5 min. Then add 3 drops of 30% H_2O_2 and heat for 10 min.

(b) Digestion:

1. The micro Kjeldahl distillation apparatus is filled with demineralized water and flushed out by allowing the water to boil until about 50 ml has collected in the receiver.

2. The lip of each flask is lightly coated with vaseline to prevent drops of liquid from running back along the outside.

3. 5-8 ml, of water are added to each flask to dissolve the solid material which is formed when the flasks cool. The contents of the flask are transferred quantitatively to the distillation apparatus (4 rinsings with about 5 ml of water).

4. 15 ml, of NaOH - $Na_2S_2O_3$ - $5H_2O$ solution are added and the funnel rinsed out with a little water.

5. The end of the receiver is placed under the surface of the liquid in a 125 ml. Erlenmeyer flask containing 5 ml, of H_3BO_3 solution and 2 drops of indicator.

6. About 25 ml, of distillate are collected.

7. The distillate is titrated with 0.02 N HCl to a
   grey-purple end point. The concentration of HCl
   may be changed to suit the needs of the type of
   sample being run.

III. Calculations:

mg. N in flask = (ml. HCl in sample - ml. HCl in blank)
(Normality HCl) x 14

VII. Thin Layer Chromatography

1) 82 cc. of double distilled water was added to 11 g.
Ecteola Resin (Macherey, Nagel and Co., Duren, Germany)
and shaken vigorously for 2 minutes. The suspension was
then poured into the applicator (DeSaga, Heidelberg, Germ-
any) and the applicator drawn smoothly across 5 glass
plates to evenly spread the suspension.

2) The plates were dried at room temperature.

3) The plates were developed in the following medium:

tertiary butanol : HCOOH : H_2O
               10   8   10   (vol/vol)

The mixture was prepared as follows: 3° butanol
was mixed with double distilled water in a 1:1 ratio, and
stored in this form.

To make 280. cc of chromatographic fluid, 200 cc.
of this mixture was mixed with 80 cc. HCOOH.

The resulting fluid was distributed evenly among 3 thin layer chromatographic tanks. The dried plates were then placed in the tanks (maximum of 2 per tank) and then withdrawn after the fluid level had reached the top of the plate.

4) The plates were dried under a fume hood at room temperature. In order to test for the presence of the enzyme, adenylate kinase (myokinase, E.C. 2.7.4.3), advantage was taken of this enzyme's resistance to boiling at pH 1 (Dixon and Webb, 1965). This treatment destroyed all other enzymes hydrolyzing ATP and ADP in the tissue, so addition of ADP to a tissue suspension should result in the formation of some ATP and AMP.

The usual methods were used to isolate the fractions, but some of the total homogenate was saved to test it for the presence of adenylate kinase. The fractions were suspended in 2-3 cc. histidine buffer, pH 1.0, and then boiled for 10 minutes. The suspensions were then incubated with 5, 10 or 20 mM ADP and sufficient $MgCl_2$ to give a 1/2 Mg/ADP ratio, the optimal ratio for the enzyme activity (Dixon and Webb, 1965). Aliquots were withdrawn at 30 and 60 minutes, placed in 15% (w/v) TCA, and centrifuged in a clinical centrifuge at maximum speed. Controls consisting of ATP, ADP and AMP with and without homogenate, but always without Mg^{++} were also run.

Commercially obtained capillary tubes were placed in
the supernatant obtained above. The fluid was allowed to rise
in the tube and 4 drops from each tube placed at each
starting point. Each drop was dried with a Master Appli-
ance Corp. heat gun, Model MG-201, before the next drop
was applied.

After the completion of spotting, the plates were
placed in the developing medium, and removed when the fluid
level was approximately 2 inches from the top.

The plates were then dried by an air blower, and then
examined under ultraviolet light. Using this same proced-
ure, the presence of adenylate kinase was demonstrated in
all subcellular fractions of rabbit aorta (Wolowyk, M.,
unpublished observations).

VIII. Electron Microscopy

1) Copper grids (100 mesh) coated with collodion or
carbon were dipped through a drop of the fraction suspen-
sion which was placed in dental wax, and the grid was
dried on filter paper.

2) While the grid was drying, one drop of OsO_4 per
grid was placed on the dental wax.

3) The grid was then immersed in the OsO_4, and fixed
for 30 minutes.

4) The grid was dried on filter paper placed on a

hot plate at 60°C for 10 minutes.

5) The grid was then dipped through double distilled water for 1 minute, and then dried on the hot plate for 10 minutes.

6) The grid was placed on phosphotungstic acid for 10 minutes, and then dried on the hot plate for 10 minutes.

IX.   Electrolyte Determinations

The fractions were isolated as usual, and the resulting pellets placed without homogenization into glass test tubes.   The tubes were then oven-dried at 105°C, and then placed in sand on hot plates at 80°C.   Concentrated HNO$_3$ was used intermittently and the temperature raised to 120°C until a white powder remained.   The powder was then dissolved in .1 N HCl, and the cation concentration determined on a Unicam SP 900 flame spectrophotometer (Unicam Instruments, Cambridge, England).   Flame emission spectrophotometry was used for Na$^+$, K$^+$ and Ca^{++}; readings were made at 589, 766 and 422 mμ, respectively.   Atomic absorption spectrophotometry was used for Mg^{++} at 285 mμ.   The μM concentration was usually determined in a volume of 25 or 40 cc, using external standard solutions.   This gave the number of μmoles contained in this volume, which equalled the number of μmoles per fraction.

A fraction of the total homogenate was retained for

electrolyte determination of the total tissue, and to pro-
vide a basis for estimating the % recovery of the ions in
subcellular fractions.

X. Composition of Phosphate Free Krebs for Intact Tissue
Studies

A. Stock Solutions

    1.   9% NaCl (90 gm. NaCl/liter)

    2.   5.75% KCl (28.75 gm. KCl/500 ml.)

    3.   8.06% $CaCl_2$ (40.3 gm. $2H_2O$ $CaCl_2$/500 ml.)

    4.   15.7% $MgCl_2$ (78.5 gm. $6H_2O$ $MgCl_2$/500 ml.)

B. Deca Krebs

        500 ml.   1

         40 ml.   2

         30 ml.   3

         10 ml.   4

C. Krebs Medium

    1. Dilute 100 ml. deca Krebs to 1 l.

    2. Add 80 ml. of 2.6% $NaHCO_3$ (26 gm/l.).

    3. Add 50 ml. fresh 20% glucose.

XI. Oxygen Uptake

The fractions were isolated as usual, with 50 mM Tris added to the .25 M sucrose isolating medium. The fractions were then suspended in the appropriate volume of polarographic buffer which consisted of .25 M sucrose, 20 mM KCl, 10 mM $MgCl_2$, 20 mM Tris and 20 mM phosphate buffer, adjusted to pH 7.4.

The change in concentration of oxygen in the reaction solution was detected as the change in cathode limiting current, and was recorded automatically with a Gilson Medical Oxygraph Model KM (Gilson Medical Electronics, Middleton, Wisc.). Each reaction cell used had a 3.0 ml capacity, and two narrow necks, one used for inserting the electrode, and the other for adding the reaction components. The reaction mixture was initially stirred, and a rapidly oscillating cathode was used throughout the assay. The concentration of oxygen in the air saturated iso-osmotic medium was 240 μM at 25°C.

2.5 ml of suspending medium without tissue was added to the chamber, and 250 lambda of suspension added. Substrate added (succinate) was in volume of 100 lambda.

Method of calculation:

Example of mitochondrial fraction.

From the calibrated paper recorder, read, 200 μM O_2/250 lambda/400 seconds.

200 μM = 200 μmoles/l = .5 μmoles/2.5 cc. bath

volume = .5 μmoles/.25 cc. tissue suspension/400

secs.

If original tissue weight was .5 g, and the final

pellet was suspended in 1 cc. suspending buffer, .25 cc.

would contain 1.605 $\div$ 4 = .4 mg N.

.5 μmoles/.25 cc. tissue suspension/400 secs. =

.185 μmoles O_2/mg N/minute.

XII. Glucose-6-phosphatase (Harper, 1963)

The tissue or subcellular fraction to be tested

was suspended in .1 M citrate buffer.

Each reaction mixture contained glucose-6-phosphate

as substrate, and buffer. The suspension was added to

the buffered substrate, incubated for 15 minutes at 37°C,

and the reaction stopped by adding a 10% (w/v) solution

of TCA. Phosphate was determined by the method of Fiske-

Subarrow. Appropriate controls were always run.

RESULTS

I. Intact Tissue

Nucleotidase activities of the myometrial cell surface were studied. A non-specific ATPase activity was found to be present, which was ouabain and Na^+ insensitive. These findings will therefore be discussed later.

II. Total Homogenate

The ATPase activity of rat myometrium was studied first in a total homogenate of longitudinal muscle. Figure 2 shows a pH-activity curve for the enzyme activity in this homogenate, using a complete medium (Mg^{++}, Na^+ and K^+. See methods). The curve is unusual in that ATPase activity was maximal over a pH range of 6.5 to 8.0. Figures 3 and 4, and Table I show the effects on the ATPase activity of adding various compounds to the assay medium. Figure 4 also shows the rate of hydrolysis of CTP and GTP.

The omission of Mg^{++} from the assay medium resulted in a 65% reduction in the ATP splitting. The residual ATPase was ouabain insensitive, but Na^+ inhibited.   The Mg^{++} concentration in the whole tissue is 5 mM/kg. wet weight (see section II), and even without addition of $MgCl_2$ to the assay medium, the medium would have a Mg^{++} concentration of about .2 mM. This may be a high enough

FIGURE 5.

MINUTES

μmoles Pi/g wet wt of tissue

1 CONTROL

2 −Na⁺ (± OUABAIN 10⁻³M)

3 +OUABAIN 10⁻²M, 10⁻³M

4 −Na⁺ −Mg⁺⁺ (± OUABAIN 10⁻³M)

5 −Mg⁺⁺ (± OUABAIN 10⁻³M)

FIGURE 4.

FIGURE 3.

μmoles Pi/g wet wt of tissue

MINUTES

μmoles Pi

10.6

9.4

8.2

7.0

5.8

4.7

3.5

2.3

1.2

CONTROL

OUABAIN 10⁻⁵ M

OUABAIN 10⁻⁴ M

OUABAIN 10⁻³ M

FIGURE 2.

μmoles Pi/g wet wt of tissue

pH
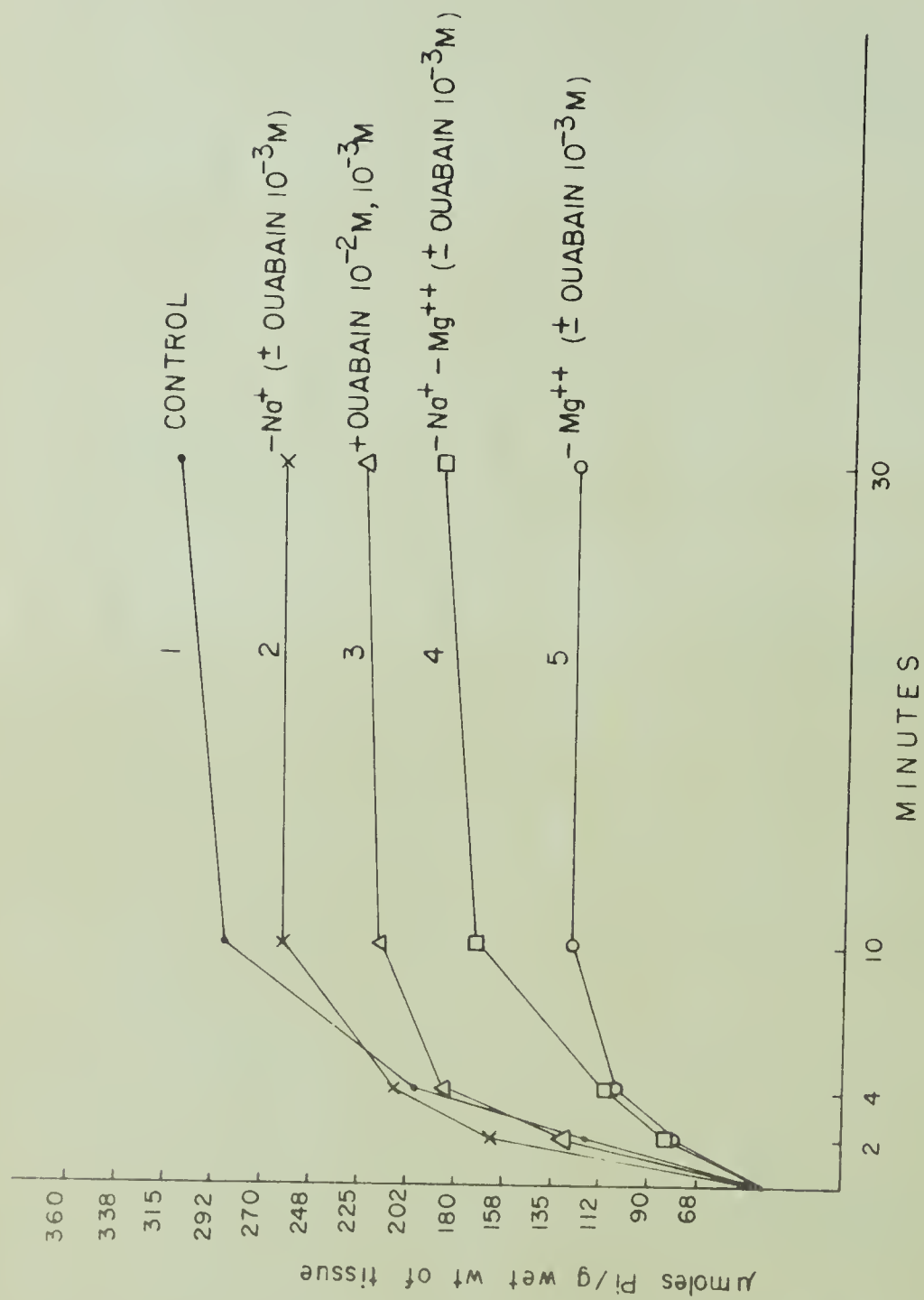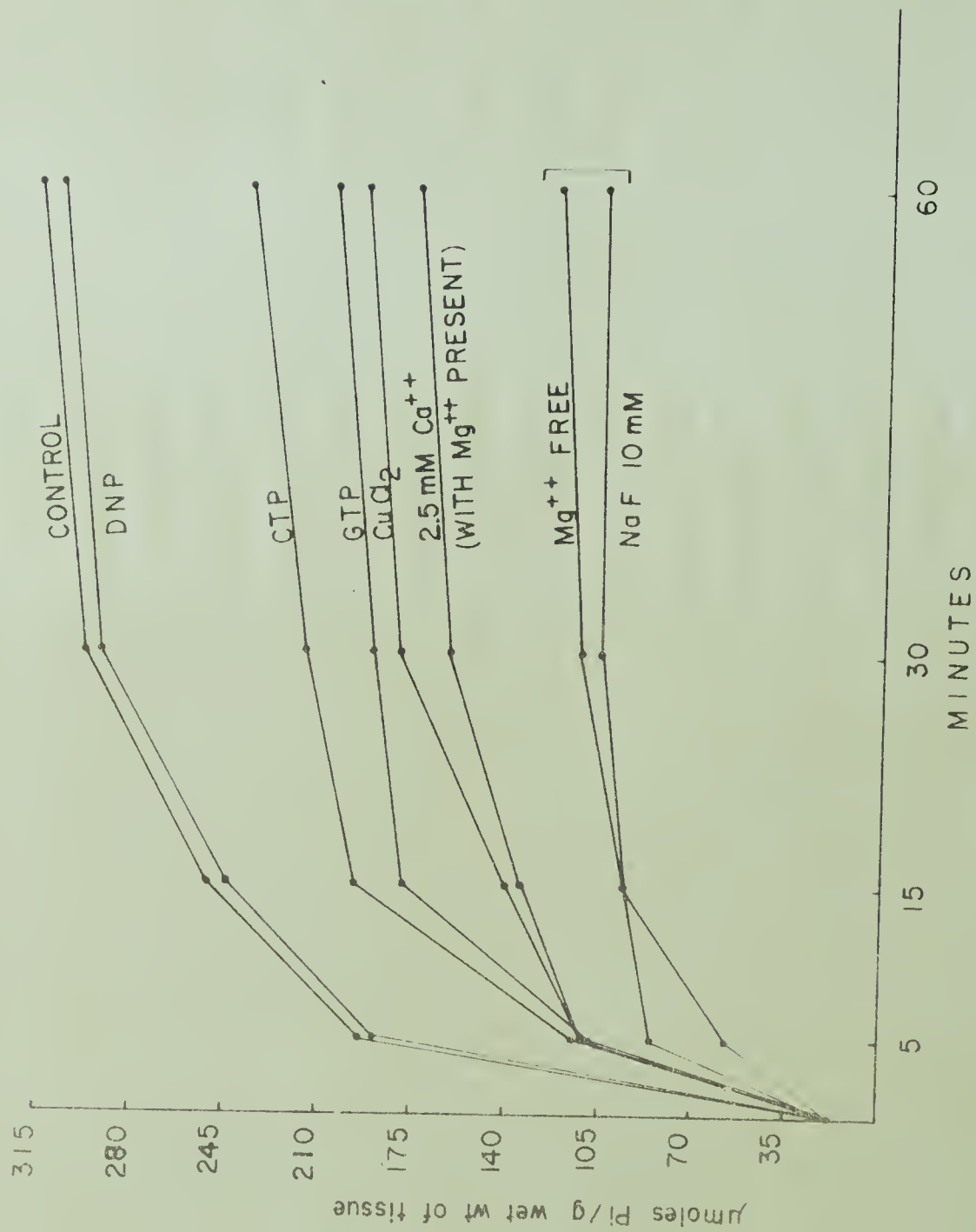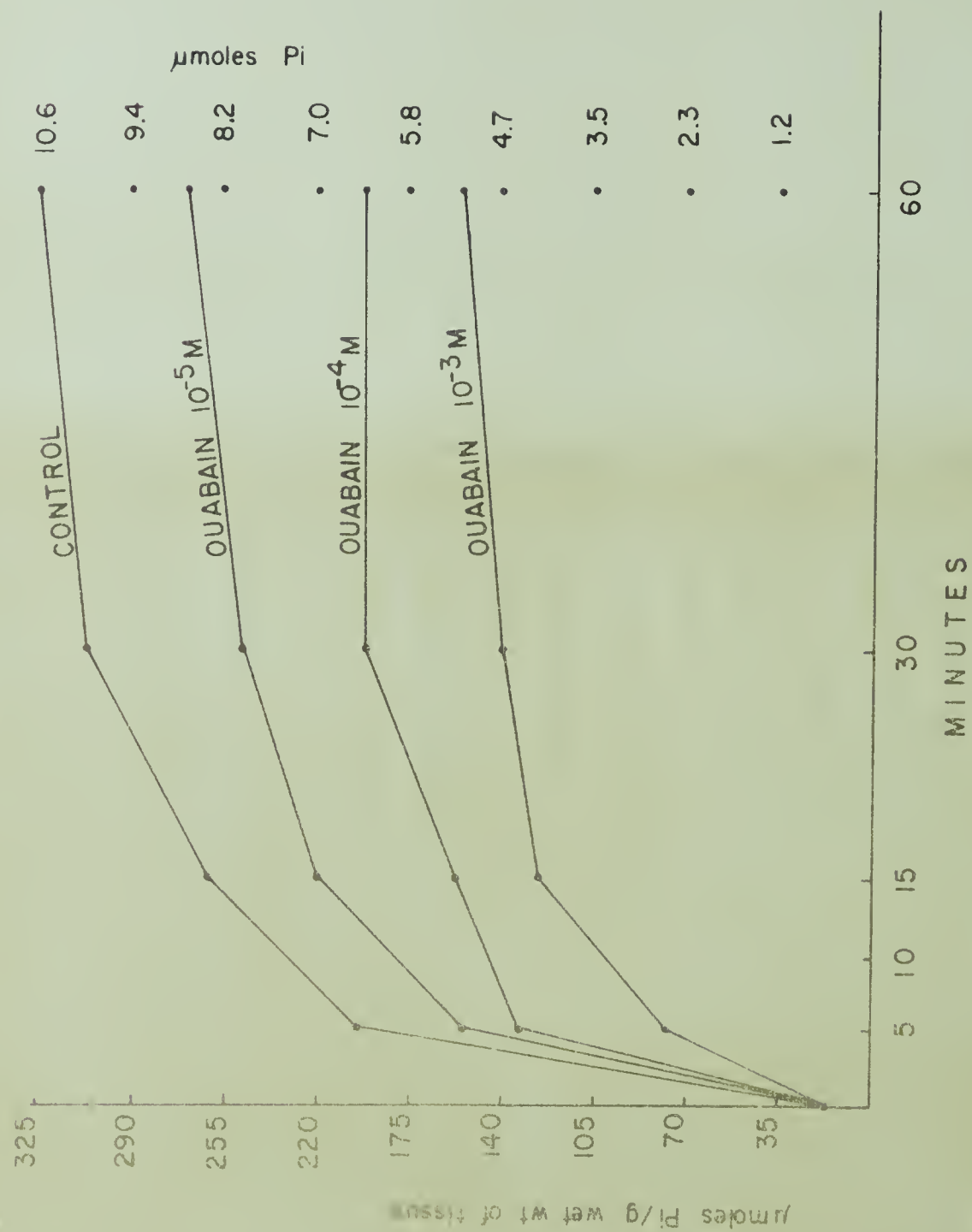
TABLE I

Medium	Time			
	5'	15'	30'	60'
Regular	193.1	263	312	352
CTP (for ATP)	121.5	216	222	243
GTP (for ATP)	108	176	182	196
Ouabain 10^{-5} M	171	214	247	267
Ouabain 10^{-4} M	147	174	194	194
Ouabain 10^{-3} M	83	140	153	171
Calcium 2.5 mM	122	146	179	194
Mg^{++} Free	60	103	122	134
NaF 10 mM	91	103	114	114
DNP 1 mM	213.3	259	297	349
$CuCl_2$ .1 mM	108	139	185	199

Table I. ATPase activity of total homogenate. Same as Figures 4 and 5. Activity in µmoles P_i/g. tissue wet wt.

Figure 2. pH activity curve of total homogenate ATPase activity. The activity was read at the pH values noted, always in complete assay media. pH was adjusted with HCl. P_i measured by FS method at 60 minutes.(N=3)

Figure 3. Effects of ouabain on ATPase activity of total homogenate. Ouabain was added to the complete assay media. Aliquots were taken at the time intervals shown for P_i determination by FS method. (N=5)

Figure 4. NTP hydrolysis and inhibitor effects on ATPase of total homogenate. Compounds were added and assayed in the same manner as Figure 3.

Figure 5. Effects of addition and omission of various compounds on ATPase of total homogenate. Same as Figures 3 and 4.

Mg^{++} concentration to explain the activity noted (subsequent studies with subcellular fractions indicate that no ATPase activity exists in media in which the Mg^{++} concentration is known to approximate zero).

Omission of all added Na^+ except for 5.0 mM accompanying CN^- (plus 5 mM KCN) did not depress the ATPase activity to the same degree as did ouabain (Figure 5, curves 2 and 3), and increased the early phase of release of P_i. This was thought at first to result from an inhibitory action of ouabain on a Na^+ independent ATPase. However, addition of 1 mM ouabain to media with low Na^+ concentration (no added Na^+) did not inhibit release of P_i (Figure 5, curve 2). This suggested that there was, in addition to a ouabain sensitive ATPase, an ATPase activity inhibited by Na^+ and insensitive to ouabain. (Figure 5, difference between curves 2 and 3.) This latter activity was found to be Mg^{++} insensitive, since the release of Na^+ inhibition by the omission of the cation occurred to at least the same degree without Mg^{++} as with Mg^{++}. This was so despite the reduction in total ATP splitting, which resulted from omission of the Mg^{++} (figure 5, difference between curves 4 and 5, compared to the difference between curves 2 and 3).

The omission of K^+ was also tried, but no effect was noted. Since the K^+ concentration in the homogenate

FIGURE 6

(CONTROL — NaF) = A

(CONTROL — CoCl$_2$) = B

(CONTROL — CuCl$_2$) = C

(CURVE I + (CURVE 4 – CURVE 5) – CURVE 2) = D

(CURVE I – CURVE 3) = (CONTROL – OUABAIN) = E

(CURVE 4 – CURVE 5) = F

Δ μmoles Pi/g wet wt of tissue

MINUTES
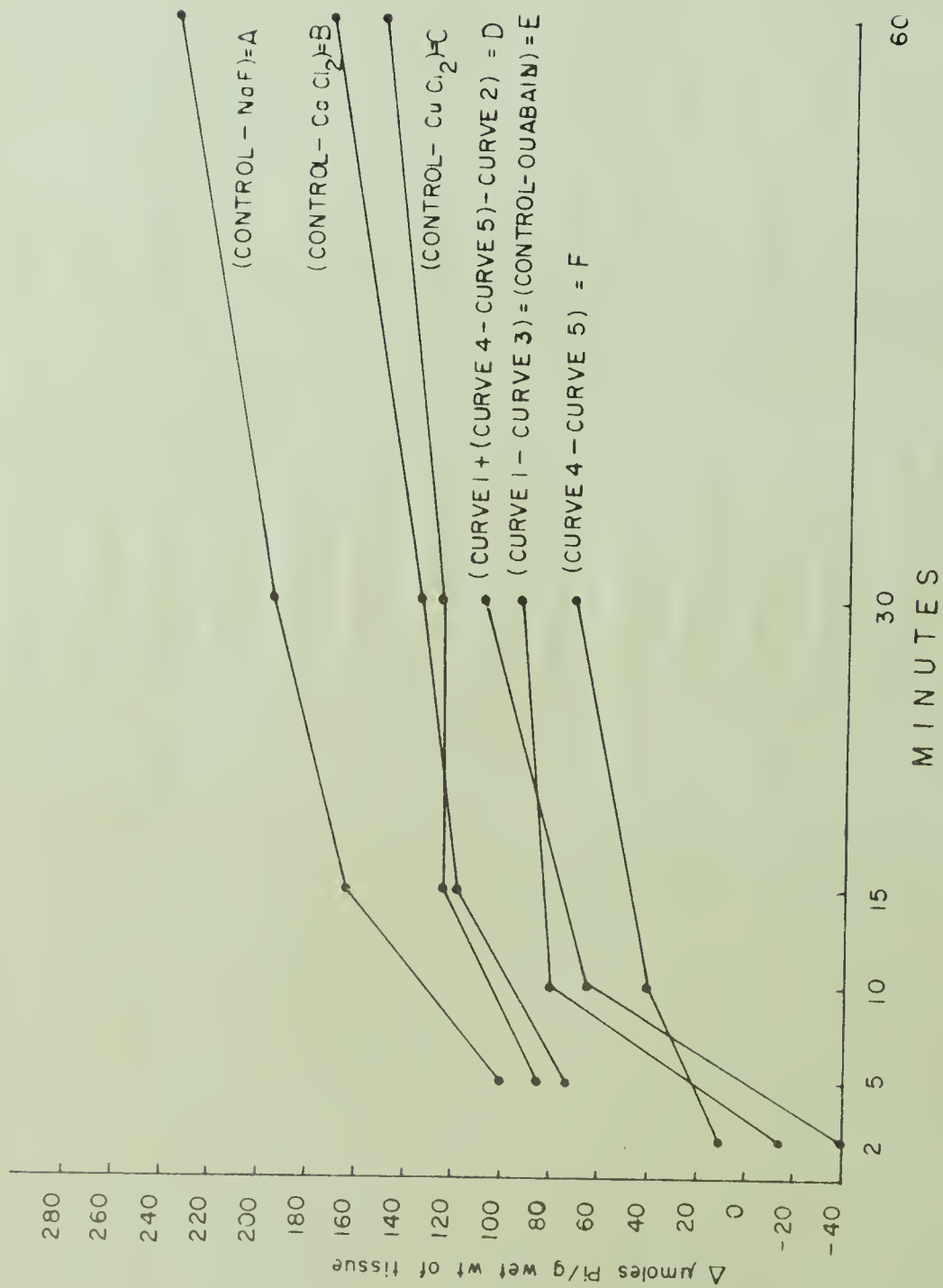
after dilution for assay was about 2 mM, the following ex-
periments were performed. Rat uteri were made Na^+ rich and
K^+ poor by exposure overnight in cold K^+ free medium, a
procedure which results in a final tissue concentration of
5-10 mmoles K^+/kg. wet weight. The diluted homogenate from
the myometria in the final assay medium contained .025 mM K.
This homogenate possessed a Mg^{++}-requiring, Na^+ activated
and ouabain inhibited ATPase like that found in homogenates
from fresh tissue (Table II). This activity was not acti-
vated by K^+ in concentrations from 2 to 100 mM. Further-
more, these concentrations of K^+ were unable to reverse
the ouabain inhibition of the ATPase activity (Table II).

Figure 6 shows some of the difference curves taken
from Figures 4 and 5. Curves A, B and C are derived from
Figure 4, and represent the following activities. Curve
A is the difference at the noted times between the activity
of the homogenate in a control (complete) medium, and the
activity remaining after the addition of 10 mM NaF. Thus
curve A is the NaF sensitive activity, and is roughly equal
to the Mg^{++} sensitive ATPase, curve B the $CaCl_2$ sensitive
activity, and curve C the $CuCl_2$ sensitive activity.

Curves D, E and F are taken from Figure 5. The Na^+
stimulated activity may be represented by curve D in Figure
6, and the ouabain sensitive activity by curve E. Curve F

Figure 6. Difference curves of various ATPase activities
    of total homogenate. This figure is a composite of
    Figures 4 and 5. Curves A, B and C are difference curves
    from Figure 4, and curves D, E and F are from Figure 5.
    Curve numbers correspond to appropriate numbers in Figure 5.

TABLE II

mM	1	2	3	4	5	6	7	8	9	10	11	12	13
2 ATP	+	+	+	+	+	+	+	+	+	+	+	+	+
5 Mg	+	+	+	+	+	+	+	+	+	+	+	+	+
80 Na	−	−	+	+	−	+	+	−	+	+	−	+	+
K	−	−	−	−	20	20	20	50	50	50	100	100	100
1 Ouab.	−	+	−	+	−	−	+	−	−	+	−	−	+
	360	340	435	285	300	430	240	360	425	300	270	419	243

Table II. ATPase activity of total homogenate left overnight in K^+ free Krebs at 0°C. Activity measured in μmoles P_i/g. tissue wet wt./hr. by LI method (N=3).

represents the Na^+ inhibited activity that appeared to be Mg^{++} insensitive. All numbers in parentheses in curves D, E and F in Figure 6 represent the numbered curves of Figure 5. The Na^+ stimulated activity was determined as indicated in Figure 6, where curve 1 equals the total enzyme activity, curve 4 - curve 5 equals the Na^+ inhibited activity which is Mg^{++} insensitive and curve 2 equals the Na^+ insensitive activity in the presence of Mg^{++}. Thus the control curve, plus the Na^+ inhibited portion, minus the Na^+ independent fraction should give the Na^+ sensitive activity if each of these activities is independent of the others. The result-ant curve (D) was similar to that for the ouabain-sensitive activity (E), and at all times after 2 minutes were larger than the Na^+ inhibited Mg^{++} "insensitive" curve (F).

This may explain why Na^+ stimulation is present (difference between curves 1 and 2, Figure 5) but is less than expected from the ouabain inhibition. It also can explain the increase in 2 minute splitting of P_i in the absence of Na^+.

In Figure 6, curve A shows that the largest inhibi-tion of enzyme activity is produced by NaF. Curves B and C suggest that $CaCl_2$ and $CuCl_2$ are in fact inhibiting the same enzymatic activity, although 2.5 mM $CaCl_2$ may not be the maximal inhibitory concentration of that ion. All other drug concentrations were maximal. Comparison of curve D (Na^+ stimulated activity) and curve E (ouabain

sensitive activity) indicate that these activities approxi-

mate each other, and ouabain seems to inhibit all the Na^+

stimulated activity.

Calculation of the difference curves D, E and F,

assumes no interaction between treatments (e.g. that Na^+

inhibition is the same in the presence and absence of Mg^{++},

and in the presence and absence of ouabain). This assump-

tion cannot be verified by the above data. However, a

comparison of the difference between curve 2 and 3, and

curves 4 and 5, Figure 5, could be used to indirectly

justify these assumptions.

$$\Delta \text{ }\mu\text{moles } P_i$$

Mins.	Curve (2-3)	Curve (4-5)
2	29	0
4	22	0
10	60	55
30	45	65

Since these values do not coincide, the assumption

of no interaction appears to be invalid.

To analyze the ATPase activity of myometrial homo-

genates, an alternative approach (addition of more than

one inhibitor to the assay medium) was tried.

FIGURE 7
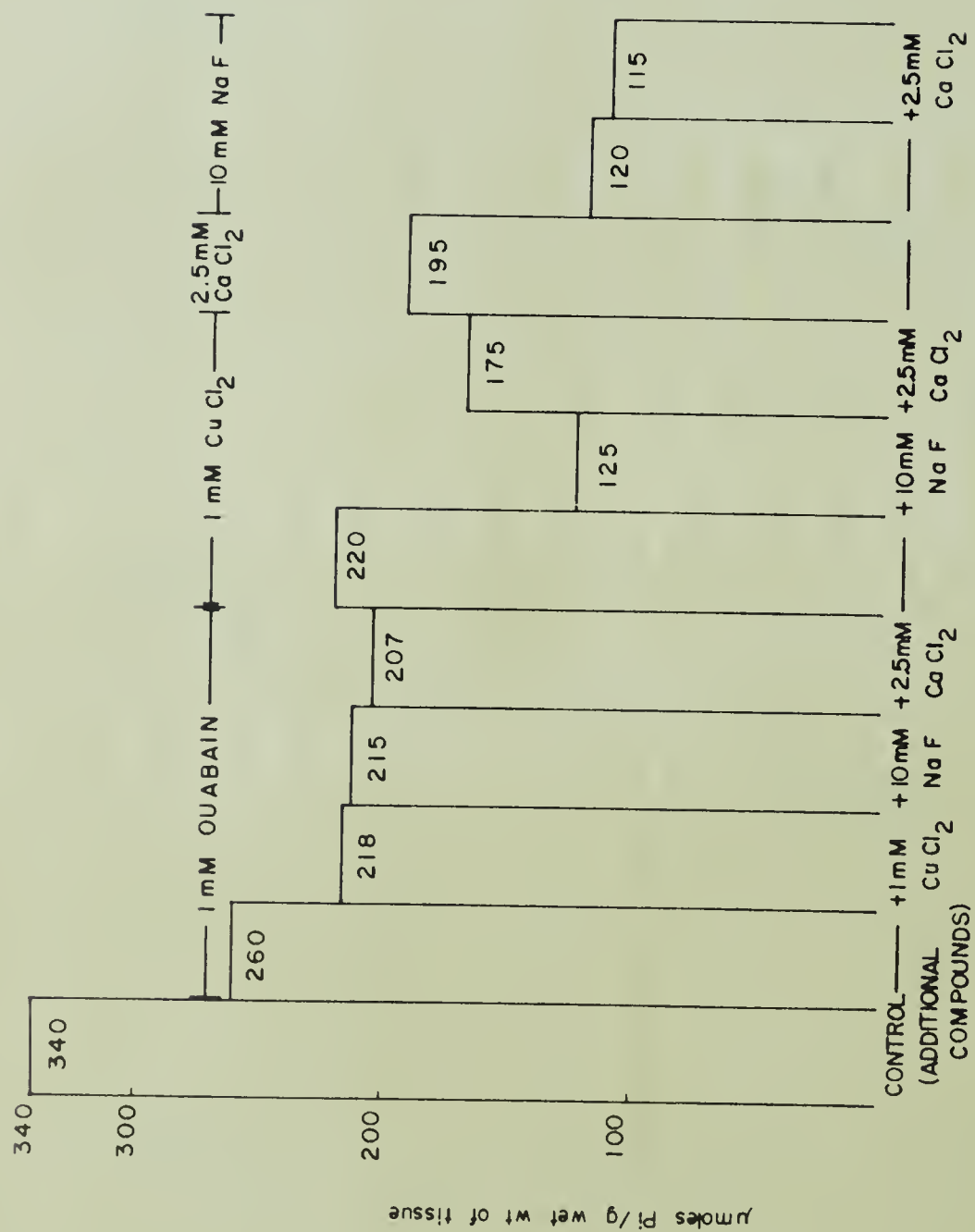
Figure 7 shows the effect of adding more than one inhibitor to the assay medium. No single inhibitor had a greater effect than did 10 mM NaF (or Mg^{++} omission, see Figure 4), and no other inhibitor added to the effect of NaF. Hence NaF inhibited all the ATPase activities inhibited by the other substances. NaF and possibly $CuCl_2$ can add to the inhibition caused by 2.5 mM $CaCl_2$, suggesting that they act on additional ATPase activity, or that 2.5 mM $CaCl_2$ is a submaximal inhibitory concentration. All other inhibitors add to the inhibitory effect of ouabain, but ouabain does not add to the inhibitory effect of any other substance . This suggests that ouabain has a site of action in common with the other substances, but that each of the other substances has an additional site of action. However, 1 mM ouabain seems to partially overcome the NaF and $CuCl_2$ inhibitory effects.

In summary, myometrial homogenates appear to contain 1) a Mg^{++} and ouabain independent, Na^+ inhibited ATPase. The reason that no added Mg^{++} is necessary for this activity could be due to the small amount of Mg^{++} present in the homogenate; 2) a Mg^{++} dependent, ouabain and Na^+ independent ATPase; 3) a Mg^{++} dependent, Na^+ activated, ouabain inhibited ATPase.

Figure 7. Effects of inhibitors on ATPase activity of total homogenate. The inhibitors indicated were added directly to the assay tubes prior to homogenate addition. A blank (-) at the bottom of the bar graph means that only the compound designated at the top of the graph was added. Aliquots were taken at 60 minutes and P_i was measured by the FS method.

Most ATPase activities but #1 can be prevented by 10 mM NaF, and this inhibition can be partially overcome by 1 mM ouabain, but not by $CuCl_2$ or $CaCl_2$. $CaCl_2$ and $CuCl_2$ inhibit the same activity as ouabain (activity #3 above), and each has an additional inhibitory effect, possibly on activity #2. $CuCl_2$ appears to add slightly to the inhibition produced by $CaCl_2$, but the latter substance may be present in a submaximal concentration.

The Mg^{++} insensitive activity appears to be the same as the NaF insensitive activity, and may be equivalent to it.

III. Characterization of Subcellular Fractions

The characterization of the 3 subcellular fractions resulting from differential ultracentrifugation was made from enzyme activities. In addition, nitrogen content, electrolyte content and oxygen uptake were measured in each fraction.

Table III indicates the basic Mg-ATPase activity in a typical experiment and oxygen uptake of the 3 separate fractions[*]. The pH of 7.4 was chosen initially because of previous work reported in the literature. (In a pH activity curve of this enzyme to be illustrated later, pH 7.4 will be shown to be in the optimal pH range.)

---

[*] Since the electrode of the Gilson oxygraph is a vibrating one, large particles such as those found in the nuclear fraction containing cell debris interfered with the attempted reading.

TABLE III

Fraction	μmoles P_i/mgN/5' (N=5)	mμmoles O_2/mgN/minute (N=3)
Nuclear	7.91	Contamination with large particles prevented reading
Mitochondrial	18.37	200
Microsomal	75.68	100
Homogenate	18.68	

Table III. Basic ATPase activities and oxygen uptake of subcellular fractions. .2 cc. of each fraction suspension was used for the enzyme assay, P_i determination by LI and FS methods. [ATP] = [Mg^{++}] = 5 mM. 50 mM histidine, pH 7.2. O_2 uptake was measured in a medium of .25 M sucrose, 20 mM KCl, 10 mM MgCl$_2$, 20 M Tris, 20 mM phosphate buffer, pH 7.4, with succinate as substrate, and adding ADP.

With added Mg^{++}, and at pH 7.4, para-nitrophenylphos-phate was only very slightly hydrolyzed by any fraction using both P_i and p-nitrophenol assays (Albers et al, 1965). There was therefore little alkaline phosphatase activity demonstrable in these fraction under these stated conditions. This activity was insensitive to K^+, or ouabain (Table IV) and 100 mM Na^+ inhibited the phosphatase activity.

Table V shows the nitrogen content and electrolyte content of each fraction. The accuracy of the electrolyte determinations was checked by adding together the absolute values for each electrolyte in all fractions, and calcula-ting the electrolyte contents per kg. wet tissue weight. These values corresponded well with values found from deter-minations of an aliquot of homogenate as well as those found in unrinsed dissected tissue, except that the Na^+ values were high compared to the latter, probably due to the con-tamination caused by homogenization in the ground glass homogenizer (see methods).

Adenylate kinase (myokinase) forms AMP and ATP from ADP (2ADP $\leftrightharpoons$ ATP + AMP). It is conceivable that an apparent ADPase activity could arise from the action of adenylate kinase on ADP to form ATP and the subsequent hydrolysis of the ATP by an ATPase.

TABLE IV

| mM | | | | | (Fresh) | (Aged) |
KCl	NaCl	Ouabain	Nuclear	Mitochon.	Microsomal	Microsomal
.2	-	-	.57	2.08	4.38	4.17
.2	-	1	.62	2.15	4.30	4.05
.2	100	-	-	2.01	2.71	2.63
2	-	-	.67	2.06	4.36	4.30
2	-	1	.52	2.04	4.30	4.20
2	100	-	-	2.00	3.72	3.65
20	-	-	.58	2.12	4.47	
20	-	1	.59	2.23	4.42	
20	100	-	-	2.17	3.71	
0	0	0	.57	2.08	4.32	

Table IV. Paranitrophenylphosphatase activity of sub-cellular fractions. .2 cc. of each fraction suspension was used, and activity was measured by measuring the appearance of paranitrophenol at 410 mμ after 10 minutes reaction time. Enzyme activities expressed as μmoles paranitrophenol/mg. N/5'. Assays contained 5 mM MgCl$_2$ and 5 mM p-nitrophenylphosphate, as well as the indicated additions.

TABLE V

Fraction	μmoles/mg.N				mgN/gm original wet wt. of tissue	(N=3)
	Ca	Mg	Na	K		
Nuclear	.05	.19	1.47	.36	4.55	(4.32,4.51,4.82)
Mitochondrial	.28	.31	4.22	.24	3.21	(3.10,3.16,3.37)
Microsomal	.53	.85	6.55	3.31	1.20	(1.07,1.14,1.39)
Supernatant	.35	.98	26.25	21.00	3.08	(2.87,3.00,3.37)
Total ions in mM per kg. original tissue wet wt. (Calculated)	2.56	4.80	109.21	71.29		
Total ions in mM per kg. original tissue wet wt. (measured from homogenate)	2.28	4.67	105.34	75.45		

Table V.   Electrolyte content and nitrogen content of sub-
cellular fractions. Mg.nitrogen was determined by the Kjel-
dahl method as described in the text. Micromoles ion/mg.N
were first measured as the absolute amount of ion in that
fraction. Knowing the weight of the tissue used, the mg.
nitrogen was calculated for the fraction, and the ion con-
tent then expressed as μmole ion/mg. N. The calculated
total ion content was calculated from the addition of the
absolute values from each fraction. The values of the
bottom horizontal column were measured directly from the
total homogenate.

9A

1μ

9B

1μ
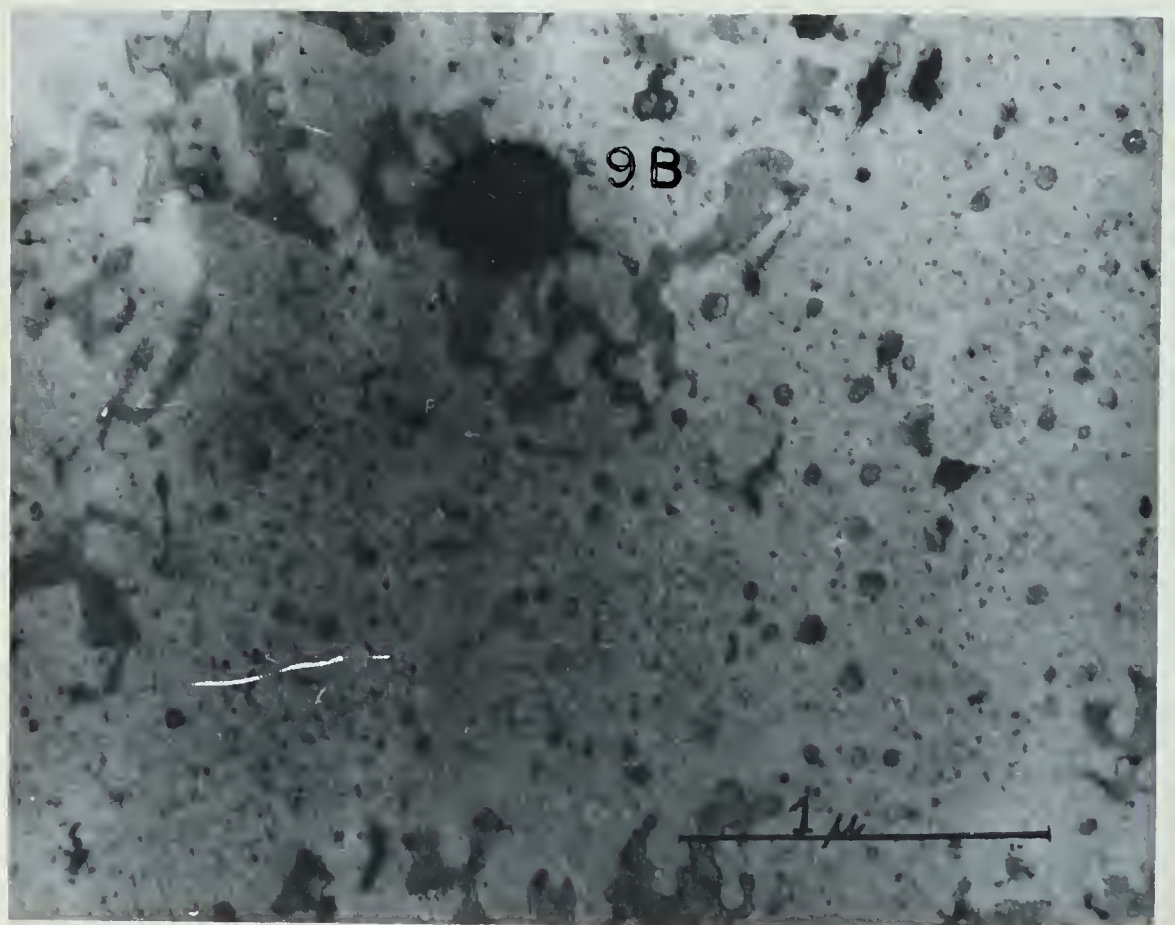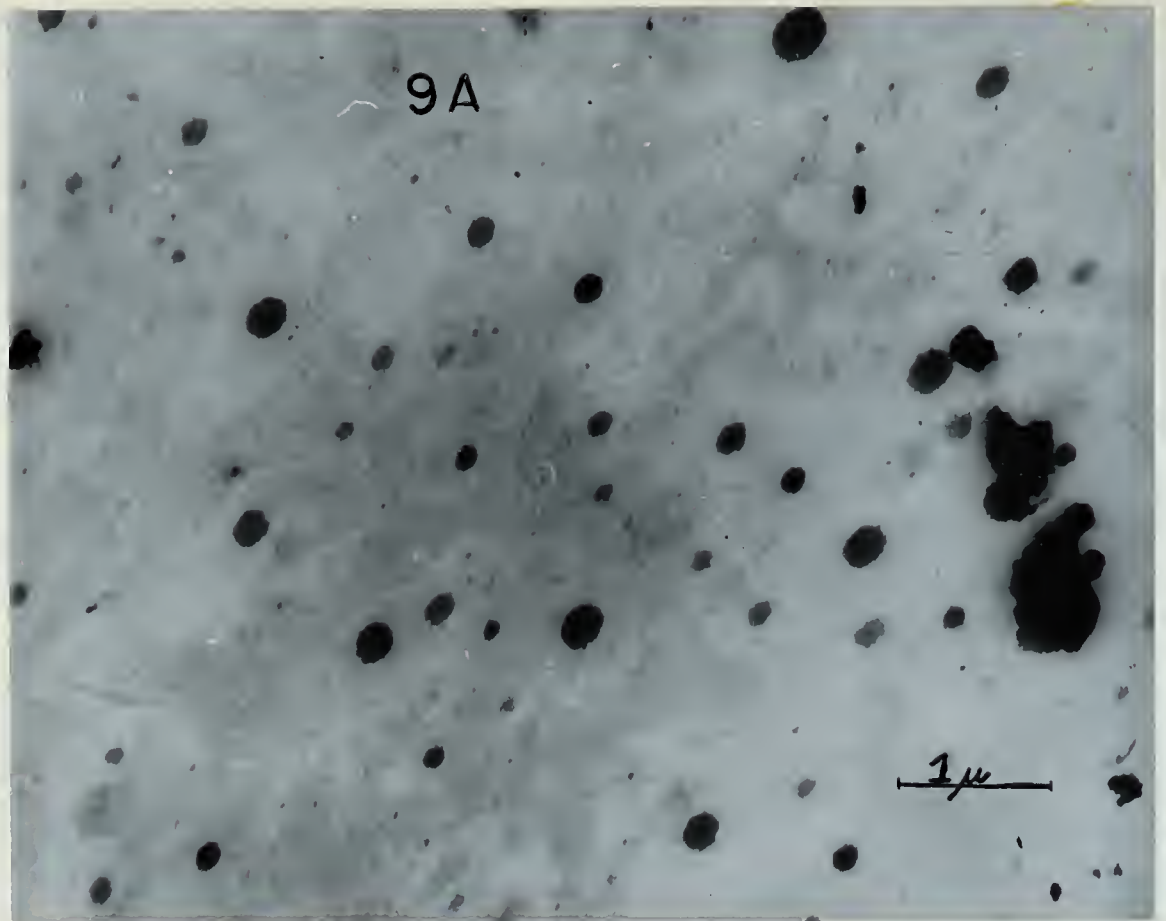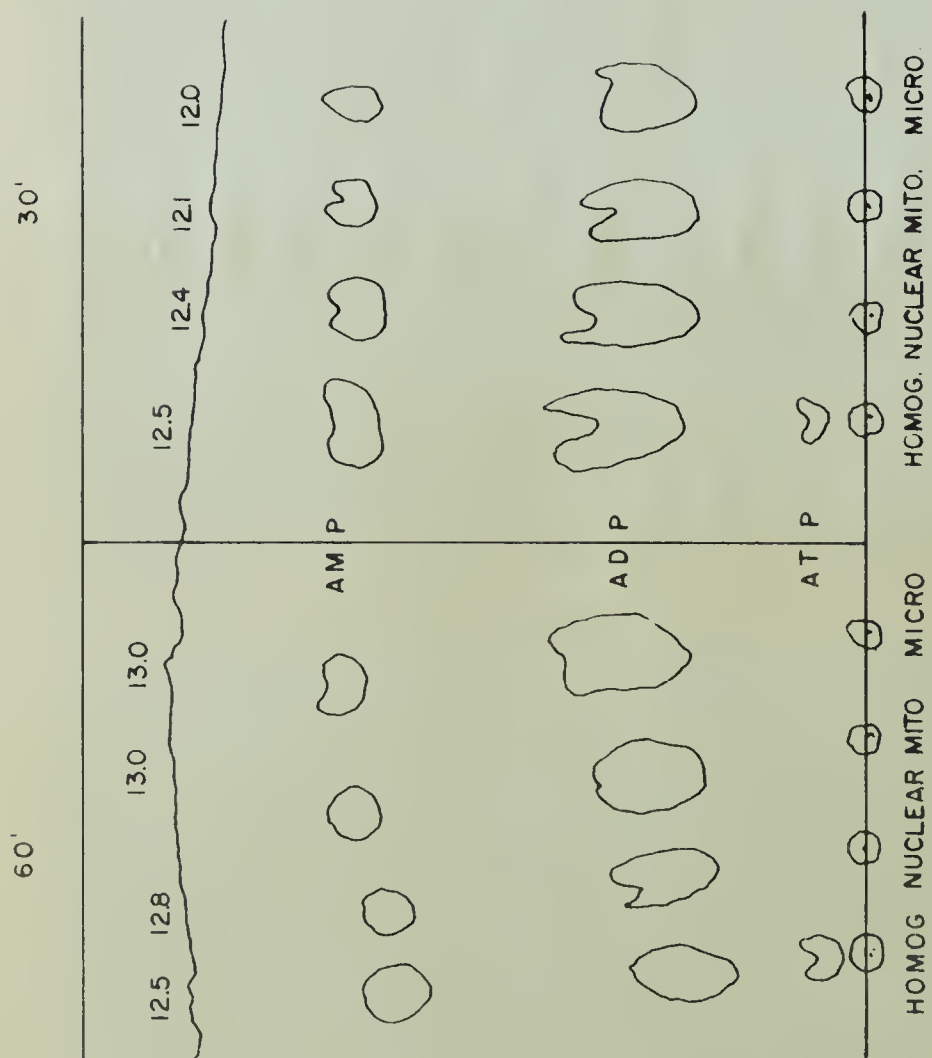
FIGURE 8

Figure 8 shows the results of an experiment repeated 4 times indicating that only very small amounts of adenylate kinase, if any, are present in the microsomal and mitochondrial fractions. The photographic representation of the chromatogram shows that only in the total homogenate is any ATP detectable, while small amounts of AMP can be seen in the homogenate as well as all 3 fractions. Since, in controls, none of the nucleotides were hydrolyzed by this procedure, as shown by control incubations, and tissue controls contained no significant quantities of nucleotides, and boiling at pH 1 for 10 minutes destroyed any ATPase or ADPase activity in the fractions, the origin of the AMP detectable on the chromatogram is unknown. However, the fact that some ATP was detectable in the total homogenate and not in the subcellular fractions can lead to the tentative conclusion that adenylate kinase is present in small quantities in the total homogenate, and that even smaller quantities are present in the subcellular fractions. Although not apparent in Figure 8, AMP spots were weakly fluorescent, and small quantities of this nucleotide may have been present.

The electron micrographs (Figure 9) merely indicate the lack of gross mitochondrial contamination of the micro-

Figure 8. Chromatogram indicating adenylate kinase of subcellular fractions and total homogenate. The R_f values of controls were the same as indicated for the experimental nucleotides. Numbers at top of chromatogram represent the distance the solvent fraction has moved in cm.

Figure 9. 9A. Electron micrograph of mitochondrial fraction. Magnification = 25,000x.
          9B. Electron micrograph of microsomal fraction. Magnification = 110,000x.

somal fraction, and the possible presence of formed vesicles in the fraction, although the oxygen uptake studies suggest a high contamination of the microsomal fraction with fragmented mitochondria. Siekivitz and others, however, (Siekivitz, 1963) have shown that relatively pure microsomes do contain oxidative activity themselves, under varying conditions.

The round appearance of the mitochondria in Figure 9 can be explained by the fact that the "drop" method used (Sjostrand, 1957), involves no embedding and sectioning of material. Thus whole mitochondria are seen, not ones which have been cut. The latter is the more familiar form, and shows the internal structure of the organelle. No evidence that nuclei contaminated this fraction was obtained.

Measurement of glucose-6-phosphatase activity in the total myometrial homogenate by the method of Harper (1961), showed the amount to be approximately 1% of that found in concurrently measured rat liver; .153 μmoles P_i/minute/g. uterus and 13.9 μmoles P_i/minute/g. liver. The only subcellular fraction of the uterus which showed measurable quantities of this enzyme activity was the microsomal fraction which had approximately .12 μmoles P_i/minute/g. original uterine weight. This enzyme is usually found in the cellular and endoplasmic reticular membrane fractions (Roodyn, 1965). Thus it can be concluded that little membranous material contaminates any of the other heavier subcellular fractions.

IV. Nuclear Fraction

In some tissues (Charnock and Post, 1963), the plasma membrane and its associated ATPase activity sediments with the nuclear fraction. As indicated in Table II, the nuclear fraction contained only a very small amount of basic Mg^{++} stimulated ATPase activity. Under no experimental conditions tried could any Na^+ or $Na^+ + K^+$ activation be noted in this fraction. All procedures which allowed Na^+ activation in the microsomal fraction were tried on this nuclear fraction (see page 124 and subsequent pages for details of these methods).

A study of the nuclear supernatant (the supernatant remaining after centrifugation of the nuclear fraction) revealed the presence of a Na^+ activated ouabain sensitive ATPase. No K^+ activation was demonstrable (Table VI). This is further evidence that the Na^+ activated ATPase did not sediment with the nuclear fraction but was left behind in the supernatant.

V. Fresh Microsomal and Mitochondrial Fractions

A. Mg^{++} Ion Requirements

It has previously been shown by other workers that all ATPases require some divalent cations for activity. The results from total homogenate studies implied that this might not be the case for myometrial tissue, since some ATPase activity existed even without the addition of

TABLE VI

5 mM ATP	+	+	+	+	+	+	+	+
5 mM MgCl$_2$	+	+	+	+	+	+	+	+
100 mM NaCl	--	--	+	+	--	--	+	+
20 mM KCl	--	--	--	--	+	+	+	+
1 mM Ouabain	--	+	--	+	--	+	--	+
	1.82	1.67	2.40	1.55	1.76	1.60	2.30	1.49

Table VI. Effects of ouabain, Na^{+} and K^{+} on the
ATPase activity of fresh nuclear supernatant.
.2 cc. of the nuclear supernatant was added to
each assay tube, with 50 mM histidine buffer,
and aliquots were withdrawn at 5 mins. for P_i
determination by the LI method. Activity is
expressed in µmoles P_i/3 cc. assay medium/5
minutes (N=5).

TABLE VI
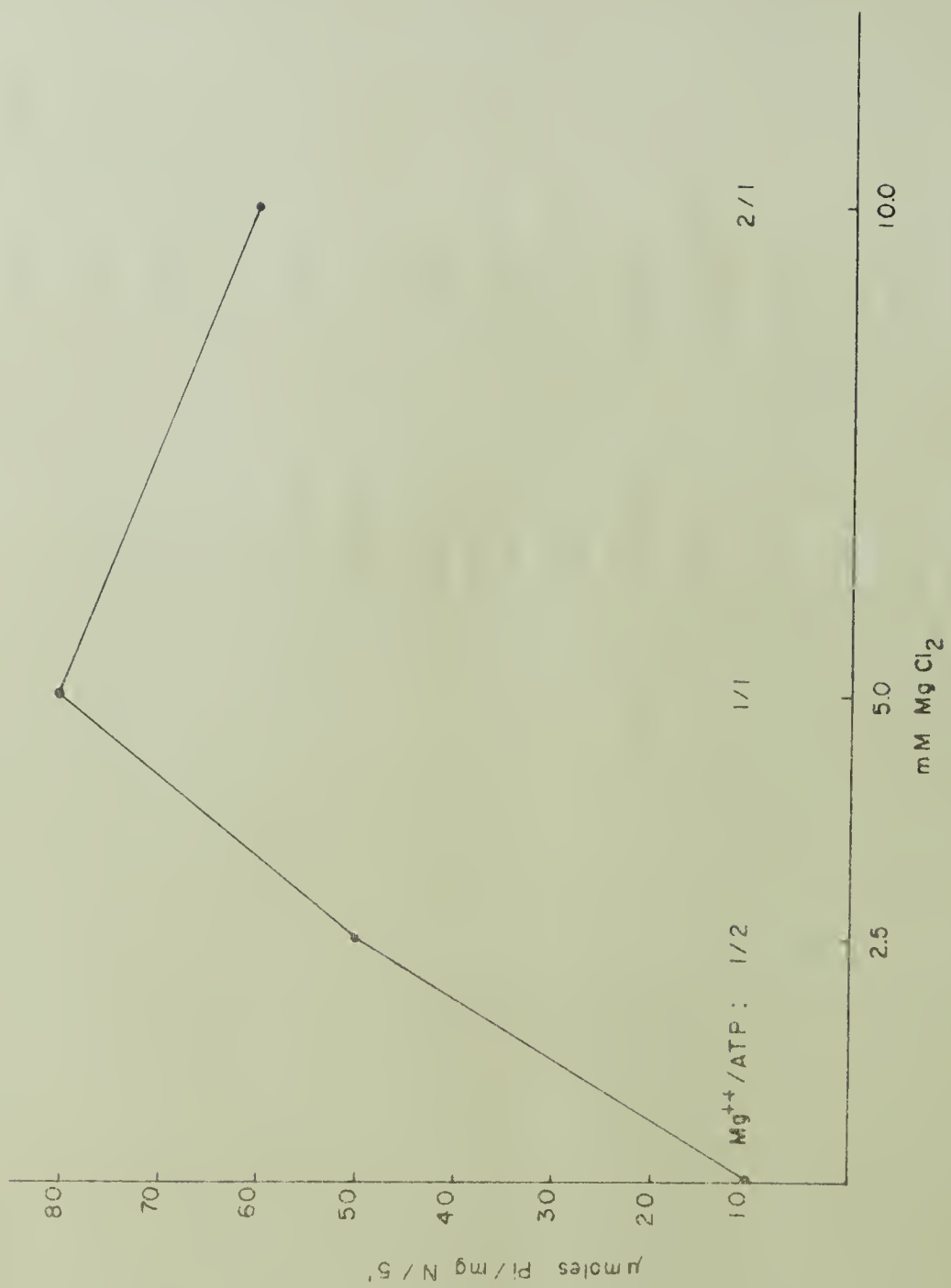
FIGURE 10

$MgCl_2$ (Figure 4). In the total homogenate experiments only .1 mM EDTA was present in the assay medium, and it is possible that residual tissue Mg^{++} could be responsible for the activation. Using the value of 4.8 μmoles Mg^{++}/kg. wet weight, 6 cc. of assay medium would contain .12 mM Mg^{++}. However, experiments with added EDTA, which should complex residual Mg^{++}, were not tried.

In any case, the Mg^{++} requirements of the microsomal fraction were examined and the results are seen in Figure 10. In the absence of added $MgCl_2$ the microsomal fraction had very little ATPase activity.

Figure 10 also shows that the Mg ATPase activity of the microsomal fraction was maximum when the Mg^{++}/ATP ratio was 1/1. Any alteration of this ratio caused a decrease in the activity of the enzymes. The dependence of ATPase activity on the Mg^{++}/ATP ratio (varied between 2 and .5) decreased quickly upon storage at 0-4°C (Table VII, Figure

---

Figure 10. Effects of altering $MgCl_2$ concentration on fresh microsomal ATPase activity. The ATP concentration was held constant at 5 mM, and the $MgCl_2$ concentration varied as indicated. Aliquots were withdrawn at 5 minutes for P_i determination by the FS method. 50 mM Tris buffer, pH 7.2 (N=4).

FIGURE II

50 mM HISTIDINE
1/1 — 2/1
N = 2

50 mM HISTIDINE
1/1 — 1/2
N = 2

50 mM TRIS
1/1 — 2/1
N = 4

50 mM TRIS
1/1 — 1/2
N = 4

(-2.82)

T (HRS) AFTER ISOLATION

△ μM Pi/ mg N / hr
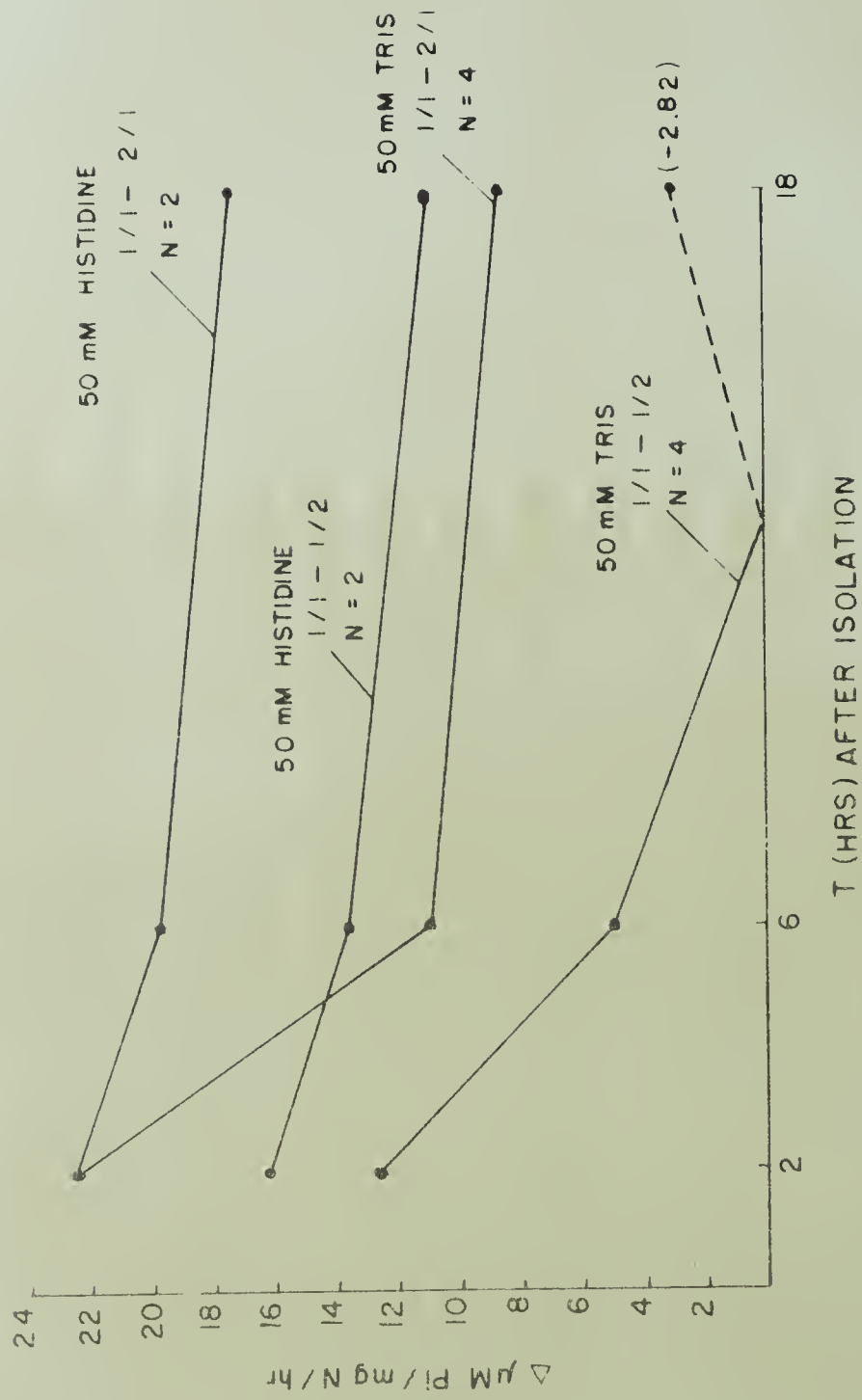
TABLE VII

	(μmoles P_i/mg. N/5') Mg^{++}/ATP			
Hours after isolation	1/2	1/1	2/1	[ATP]
2	42.12	64.56	51.96	5 mM
6	48.66	59.88	54.78	"
18	57.12	65.52	68.34	"

Table VII. Effects of Mg^{++}/ATP alterations on micro-
   somal ATPase. The microsomal fraction was suspended
   in 50 mM Tris buffer, and enzyme activity was measured
   at the indicated times after isolation, using Tris
   in the assay medium, pH 7.2. Aliquots were withdrawn
   at 5 minutes, and P_i determined by the FS method (N=
   4).

11). After storage at this temperature for 6 or more

hours, a Mg^{++}/ATP ratio of 2/1 resulted in nearly as

active an enzyme as with a 1/1 ratio. Both activities

were higher than with a 1/2 ratio. The type of buffer

used in the system seemed to make no difference in the

Figure 11. Decrease in sensitivity to Mg^{++}/ATP ratio
   of fresh microsomal ATPase. The lines marked
   histidine were stored and assayed in 50 mM histidine,
   and the lines marked Tris were treated as in Table
   VII. The curve labelled 1/1-2/1 shows the difference
   in activity between assays done at these 2 different
   ratios. The curve labelled 1/1-1/2 shows the differ-
   ence between assays done at these 2 ratios. The
   dotted line indicates that at 18 hours after isolation
   the 1/2 ratio activity actually was slightly larger
   than the activity at the 1/1 ratio.

FIGURE 12
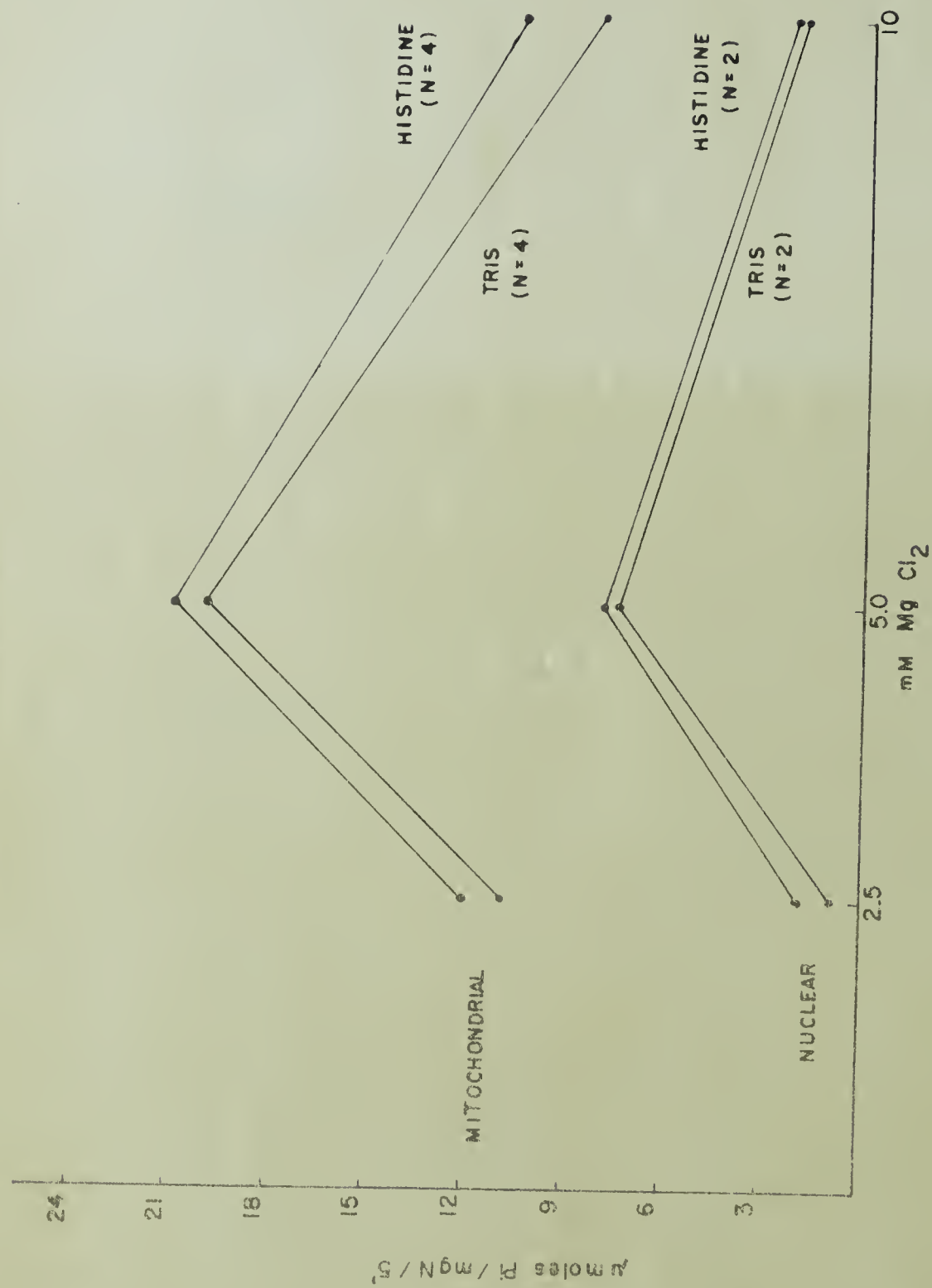
optimal Mg^{++}/ATP ratio in fresh microsomal material. Figure 11 shows that the sensitivity of the activity to the Mg^{++}/ ATP ratio persisted in histidine longer than in Tris buffer. The optimal Mg^{++}/ATP ratio was also determined for the fresh mitochondrial and nuclear fraction, and the results are given in Table VIII and Figure 12.

TABLE VIII

Fraction	μmoles P_i/mg.N/5' Mg/ATP Ratio			
	2/1	1/1	1/2	
Mitochondrial	10.71	19.75	8.69	ATP = 5 mM
Nuclear	1.08	7.38	2.11	

Table VIII. Mg^{++} requirements of nuclear and mitochondrial fractions. Same as Table IV (N = 2).

As in the microsomal fraction, the use of histidine or Tris buffer made no difference in the optimal Mg^{++}/ATP ratio, although the activity seemed to be slightly less in Tris than in histidine.

Figure 12. Effects of altering $MgCl_2$ concentrations on fresh mitochondrial and nuclear ATPase. Same as Figure 10.

FIGURE 13
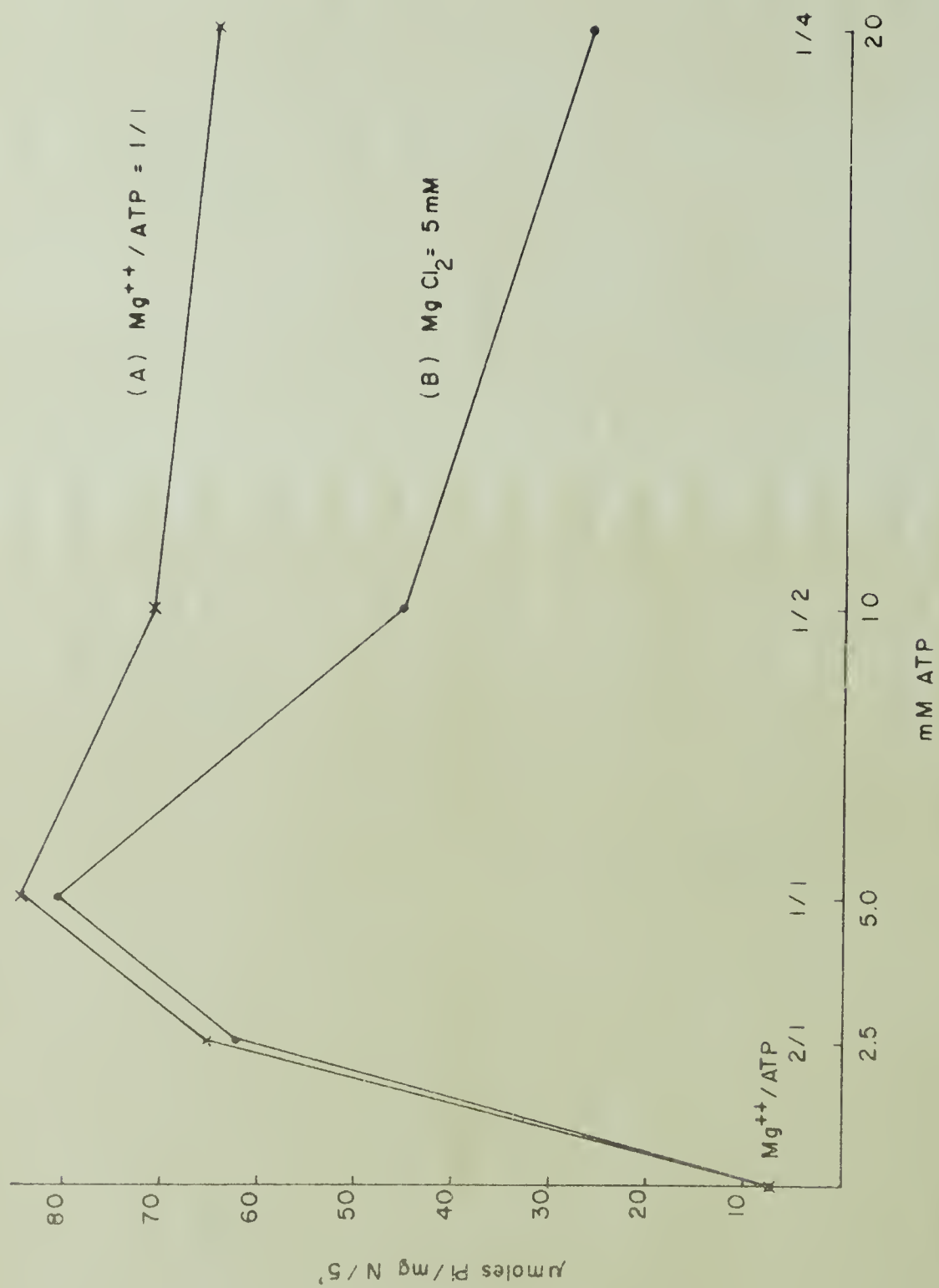
The effects of alterations of the Mg^{++}/ATP ratio on the activity of the fresh microsomal fraction were also studied by varying the ATP concentration as shown in Figure 13. This figure shows that even when the Mg^{++}/ATP ratio was maintained at 1, curve A, increasing the Mg^{++} and ATP concentrations together caused less enzyme activity (excess Mg ATP levels). This inhibition was less than that caused by increasing the ATP concentration in the presence of a constant Mg^{++} concentration curve B (excess ATP), so ATP appears to be a better inhibitor than Mg ATP at relatively high substrate concentration (> 5 mM). Figure 10 shows that increasing the Mg^{++} concentration in the presence of constant ATP (excess Mg^{++} at normal ATP levels) caused less inhibition than excess ATP levels alone but more than excess Mg ATP (comparing Figure 10 to Figure 13).

Decreasing the ATP concentration with constant Mg^{++} (Figure 13, curve B, giving excess Mg^{++} at low ATP levels) caused similar inhibition to decreasing MgATP, Figure 13, curve A (low Mg ATP levels), but not as much as decreasing Mg^{++} at normal ATP levels, Figure 10 (excess ATP, low Mg^{++}).

Figure 13. Effects of altering ATP concentrations on fresh microsomal ATPase. The ATP concentration was varied as indicated. In the top curve (A) the Mg^{++} concentration was altered to maintain an Mg^{++}/ATP ratio of 1/1. In the bottom curve (B) the Mg^{++} concentration was kept constant at 5 mM. Aliquots were taken at 5 minutes, and P_i determined by the FS method. A,N = 4. B,N = 6.

FIGURE 14
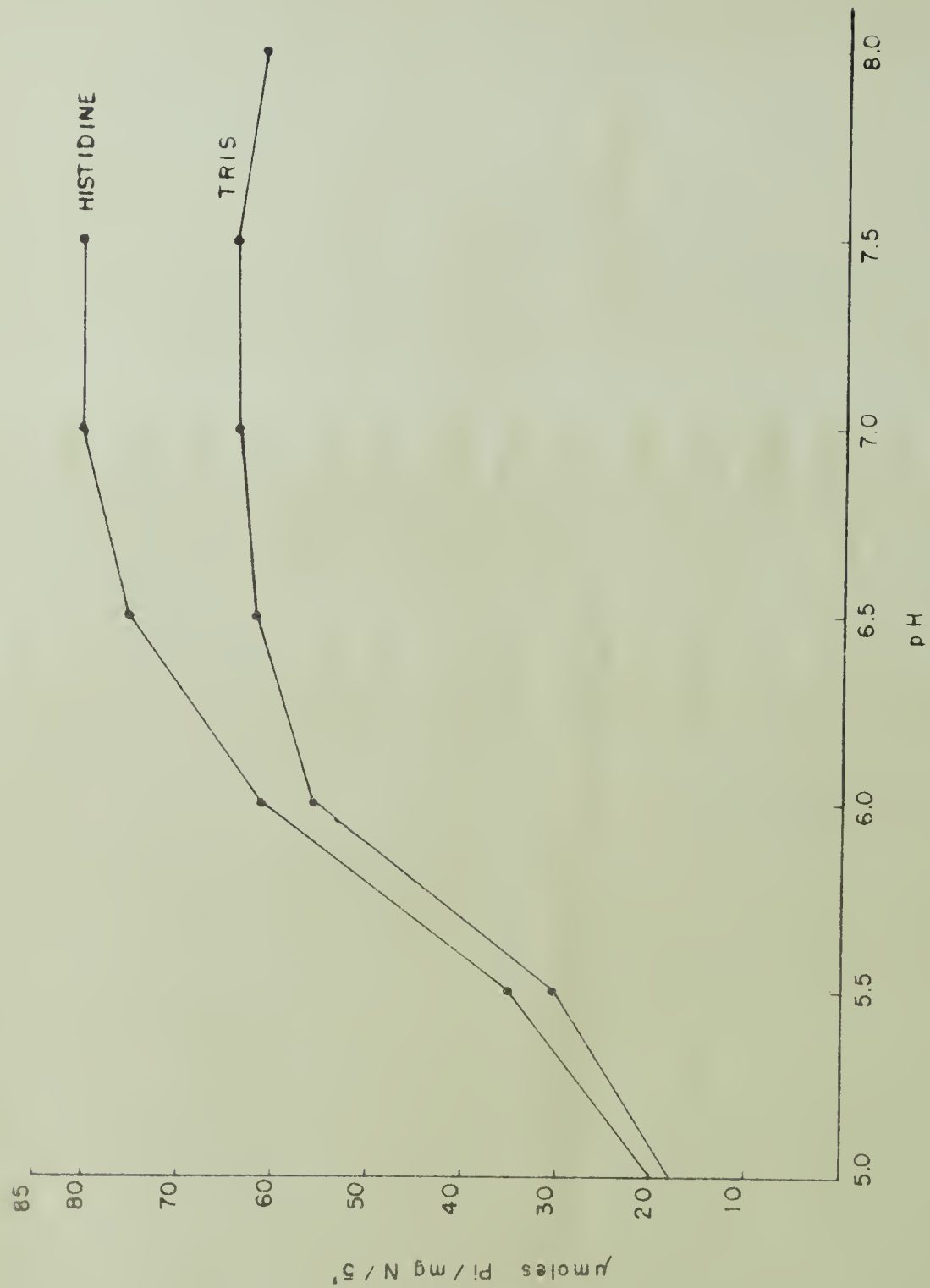
Thus it appears that ATP itself is a more potent inhibitor of the enzyme activity than Mg ATP or Mg^{++}. However, the inhibitory effect of excess Mg^{++} may be due wholly, or in part, to limited substrate availability (the substrate is presumed to be Mg ATP) by formation of the non-hydrolyzable complex Mg_2ATP.

B. pH-activity Curve

The pH-activity curve of microsomal material is presented in Figure 14, in both 50 mM Tris and 50 mM histidine buffers. A comparison with the pH curve of the homogenate (Figure 2) is in order. In histidine buffer there was slightly higher enzyme activity than in Tris. Both buffers gave unusually broad pH ranges. It should be noted that a solution of 50 mM histidine gives a maximum pH of 7.8, and base would have to be added to raise the pH. A solution of 50 mM Tris gave a pH of 10.05. Both buffers were adjusted with HCl. The results of the experiments shown in Figure 14 justify the use of pH 7.0-7.5 for the enzyme assay. The pH range in which mitochondrial ATPase had maximal activity was also 7.0-7.5.

Figure 14. pH activity curve of fresh microsomal ATPase. Usual assay procedure, using the LI method for P_i determination. Upper curve was done with 50 mM histidine (N=3) buffer, and lower with 50 mM Tris (N=4). pH values were adjusted with HCl. The presence or absence of Na^+ and K^+ made no difference in the pH-curve, although the activity was slightly lower in Tris in the presence of the ions.

FIGURE 15
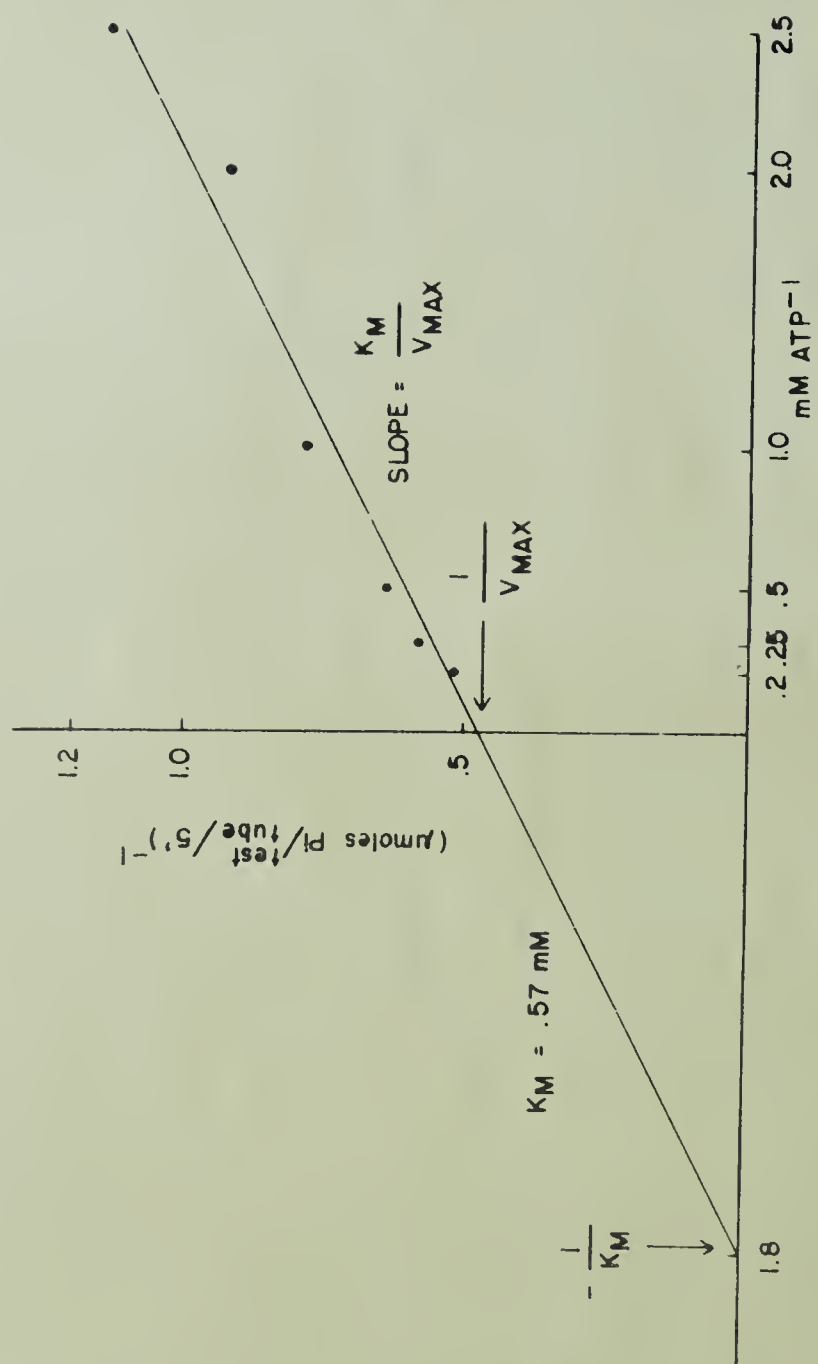
C. Determination of K_M

K_M, the Michaelis constant, has been used to characterize the affinity of a substrate for the enzyme, and may be defined as the concentration of substrate at which the reaction velocity is half-maximal. However, the assumptions applicable to soluble enzymes, and the first order kinetics upon which the calculation of K_M is based may not apply to particulate material. Despite this, the K_M may still provide a useful basis for comparison of this enzyme with particulate ATPases. The conventional Lineweaver-Burk plot was used (Figure 15), and the K_M for the basic Mg ATPase activity found to be .57 mM.

D. Hydrolysis of Other Substrates by ATPase

The hydrolysis of other nucleotides by both microsomal and mitochondrial enzyme systems was tested. The results are listed in Table IX.

In both the microsomal and mitochondrial fractions the 5 trinucleotides are all rapidly hydrolyzed by each enzyme system. This indicates a lack of specificity in the Mg^{++} stimulated NTPase activity.

Figure 15. Lineweaver-Burk plot for K_M determination (double reciprocal plot). Usual assay procedure was used with LI method for P_i determination. ATP concentrations ranging from .4 mM to 5 mM were placed in test tubes with an equivalent amount of $MgCl_2$ to maintain a Mg^{++}/ATP ratio of 1/1. The K_M value was determined from the x-intercept of the resulting curve $(-\frac{1}{K_M})$ and from the slope of curve $(\frac{K_M}{V_{MAX}})$, where V_{MAX} = maximal enzyme velocity (N=2).

FIGURE 16
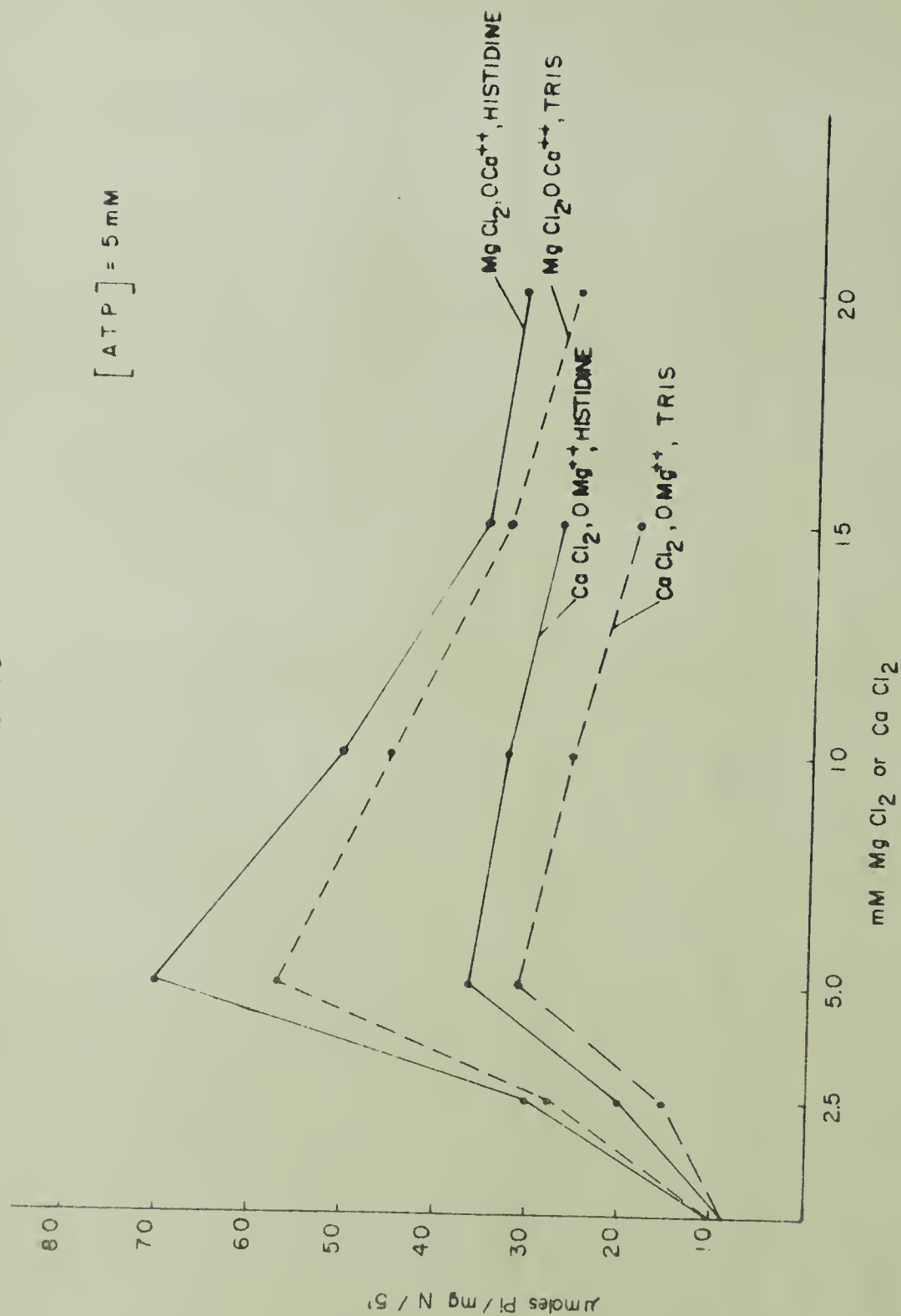
TABLE IX

Nucleotide	Mitochondrial	Microsomal
ATP	100	100
ADP	55	44
AMP	0	2
CTP	148	135
GTP	142	120
ITP	132	140
UTP	157	157

Table IX. Relative hydrolysis of various substrates by
mitochondrial and microsomal ATPases. Assays of the
activities of each fraction were carried out with .2
cc. of the subcellular suspension. ATP and Mg^{++} con-
centrations both 5 mM. Control (100) microsomal ATPase
activity was 88.4 μmoles P_i/mg.N/5'. Control (100)
mitochondrial ATPase activity was 25.3 μmoles P_i/mg.N/5'.
P_i determinations by LI method.

E. Ca^{++} Requirements

Interest in the role of Ca^{++} in smooth muscle led to

a study of the effects of this cation on the ATPase of the

microsomal and mitochondrial fractions. In Figures 16 and

Figure 16. Mg^{++} and Ca^{++} effects on ATPase activities of
fresh microsomal ATPase. The top 2 curves show the ATP-
ase activity with varying Mg^{++} concentrations and OCa^{++}.
The bottom 2 curves show the ATPase activity with varying
Ca^{++} concentrations and 0 Mg^{++}. The solid lines are with
50 mM histidine, pH 7.2, and dotted lines with 50 mM Tris,
pH 7.2.

FIGURE 19
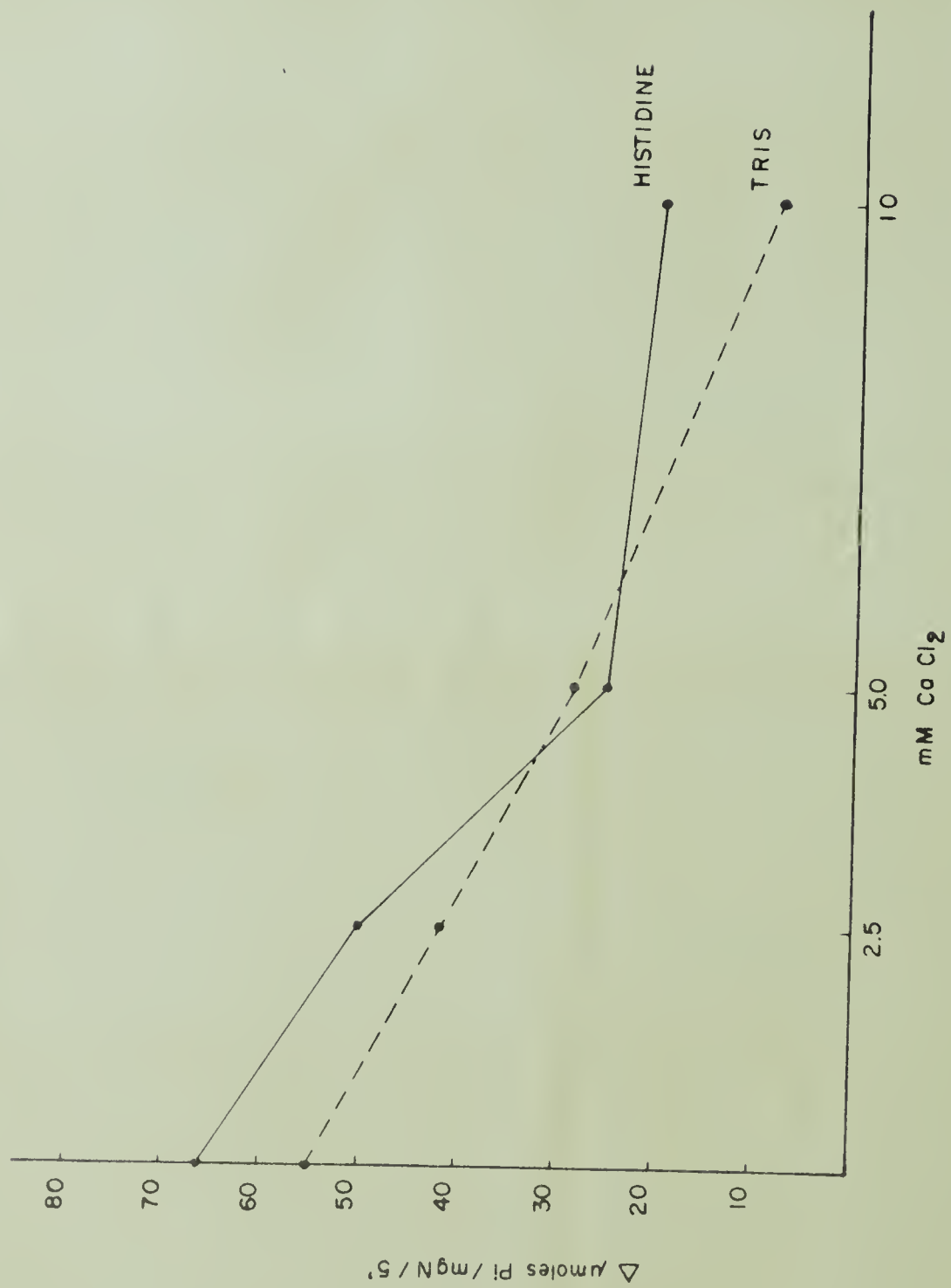
FIGURE 18
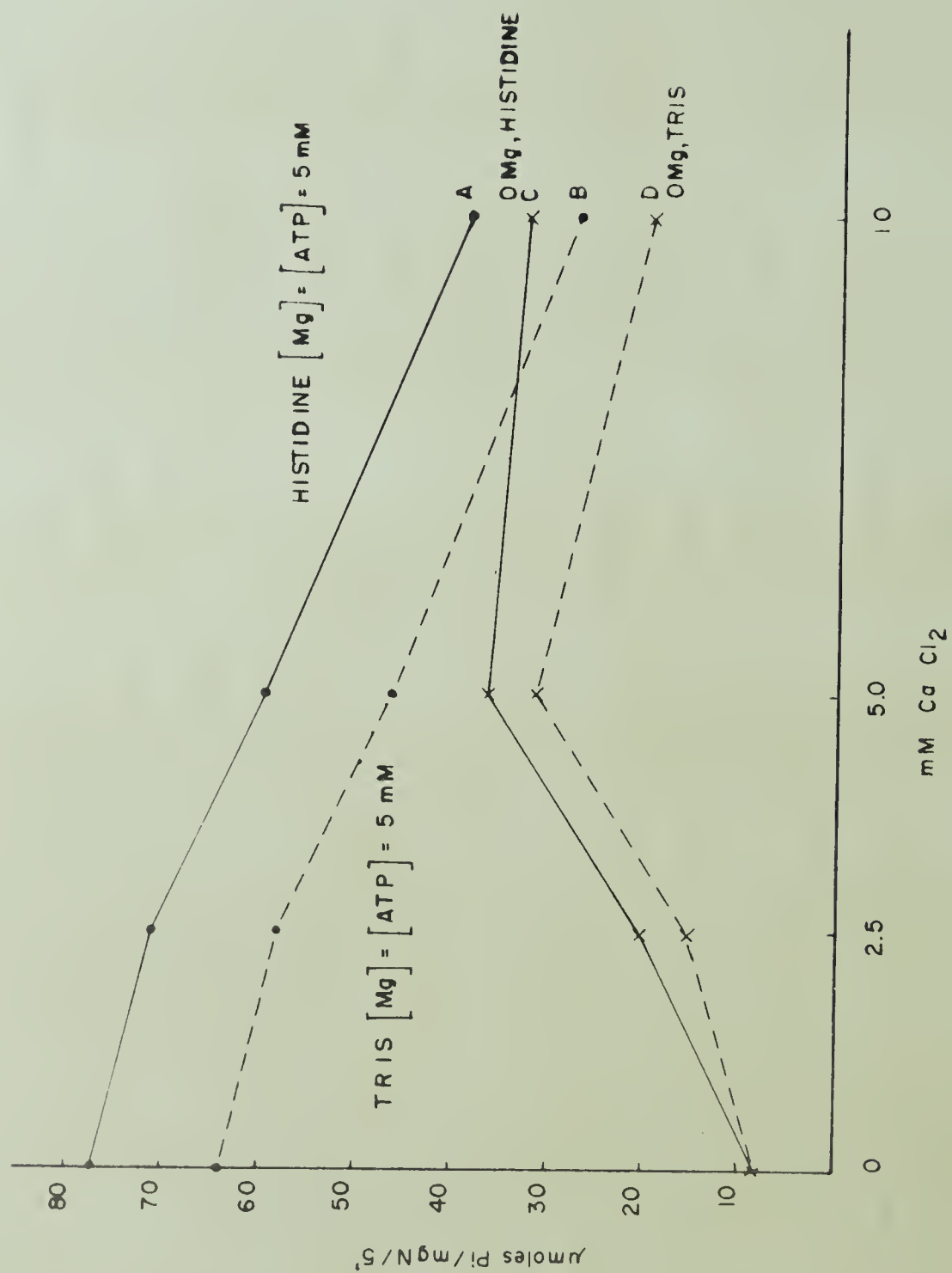
FIGURE 17
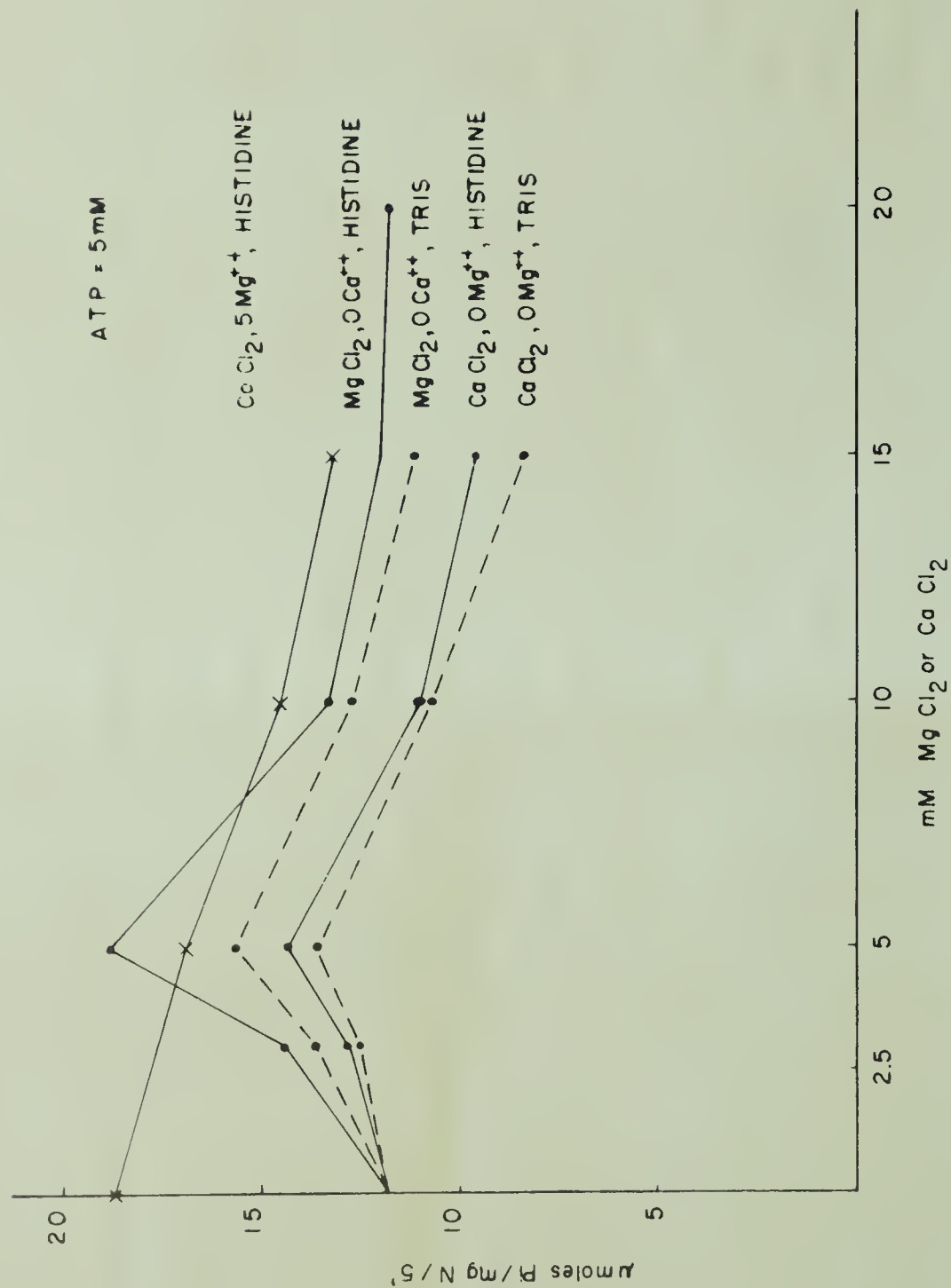
17, the divalent ion in the assay medium was either Ca^{++} or Mg^{++}. The data show that Ca^{++} can activate ATP hydrolysis in the absence of Mg^{++}, in both fractions, and that the optimal Ca^{++}/ATP ratio was also 1/1.

In the microsomal fraction, Ca^{++} caused inhibition of the enzyme in the presence of $MgCl_2$ (Figure 18). This also occurred in mitochondrial ATPase (data shown in Figure 17). It should be mentioned here that 10 mM NaF also causes inhibition of the basic Mg ATPase activity. This effect will be discussed later in this section.

Figure 19 shows the difference curves between assays with and without $MgCl_2$ at various Ca^{++} concentrations for the microsomal Mg ATPase activity. The curves show that

---

Figure 17. Mg^{++} and Ca^{++} effects on ATPase activities of fresh mitochondrial fractions. Same procedure as Figure 16. Top curve (x-x-x) is inhibition of Mg ATPase by Ca^{++}.

Figure 18. Effects of Ca^{++} on microsomal ATPase activities in the presence and absence of Mg^{++}. Ca^{++} was added to the media containing 5 mM $MgCl_2$ and 5 mM ATP. Aliquots were withdrawn at 5 minutes for P_i determination by the FS method.

Figure 19. Difference in ATPase activities in the presence and absence of $MgCl_2$. Mg ATPase activity in the presence of Ca^{++}. Derived from Figure 18. Curve marked histidine represents curve A - curve C of Figure 18. Curve marked Tris represents curve B - curve C of Figure 18.

even at 10 mM Ca^{++}, Mg ATPase probably contributes some
small amount to the total ATPase activity.

Since the mechanism of Ca^{++} inhibition of the ATPase
activity in the presence of Mg^{++} is not readily apparent,
this effect was studied in more detail in the microsomal
fraction.

The mechanism of Ca^{++} activation and inhibition of
the microsomal ATPase activity in the absence of Mg^{++}
(Figure 12) may be similar to the mechanism of Mg^{++} acti-
vation and inhibition.

Using the reiteration method of Nanninga (1961),
the MgATP, CaATP, free Mg^{++}, free Ca^{++} and free ATP con-
centrations were calculated in a series of experiments
designed to alter each of these. These results, and
the enzyme activity expressed as the absolute number
of μmoles P_i split/test tube (3 cc.)/5' are presented
together in Table X.

TABLE X

| Additions to Medium (mM) | | | Calculated Concentrations | | | | | |
ATP	Mg	Ca	MgATP	CaATP	Free Mg	Free Ca	Free ATP	Activity
2.5	0	2.5		2.2		.3	.3	1.10
2.5	0	5.0		2.5		2.5		1.21
5.0	0	2.5		2.5			2.5	1.34
5.0	0	5.0		5.0				1.62
2.5	5	0	2.45		2.55		.05	1.31
2.5	5	2.5	2.02	.480	2.98	2.02		.91
2.5	5	5.0	1.71	.790	3.29	4.21	.20	1.08
5.0	5	0	4.51		.49		.49	2.23
5.0	5	2.5	3.67	1.33	1.33	1.33	.09	1.82
5.0	5	5.0	3.13	1.87	1.87	3.13		1.35
10.0	5	0	4.95		.05			1.50
10.0	5	2.5	4.95	2.48	.05	.02	2.57	1.38
10.0	5	5.0	4.57	4.94	.43	.06	.49	.89
20.0	5	0	4.92		.08		15.08	1.80
20.0	5	2.5	4.85	2.45	.15	.05	12.70	1.01
20.0	5	5.0	4.70	4.93	.30	.07	10.37	.81

Table X. Variation of microsomal ATPase activity with calculated concentrations of various assay components. Mg^{++} concentrations were either 0 or 5 mM, and ATP and Ca^{++} concentrations were varied as indicated. mM concentrations of the other components were calculated. Aliquots were taken at 5 minutes for P_i determination by LI method (N=4). Activity is in μmoles P_i/test tube 3 cc./5'.

FIGURE 21
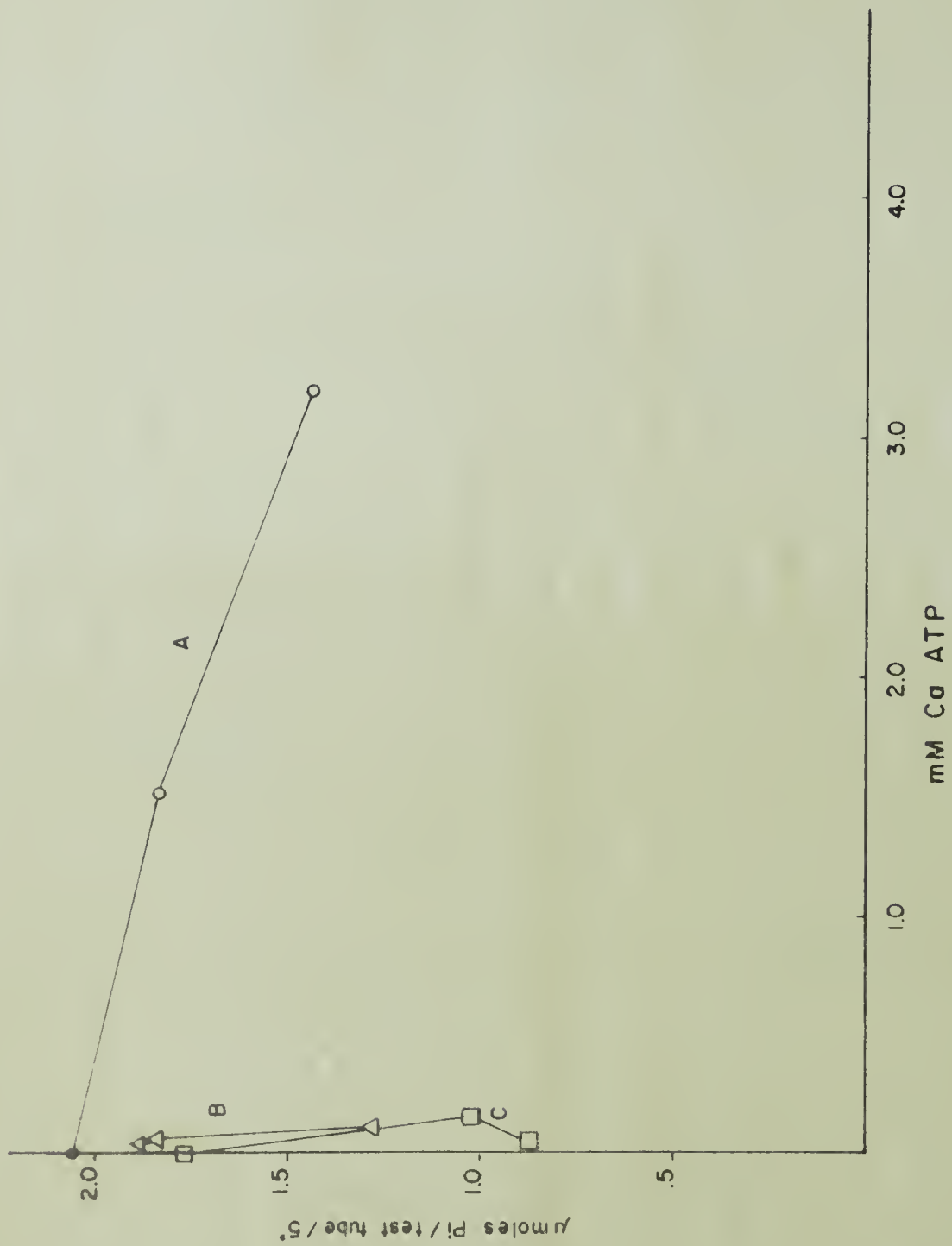
FIGURE 20
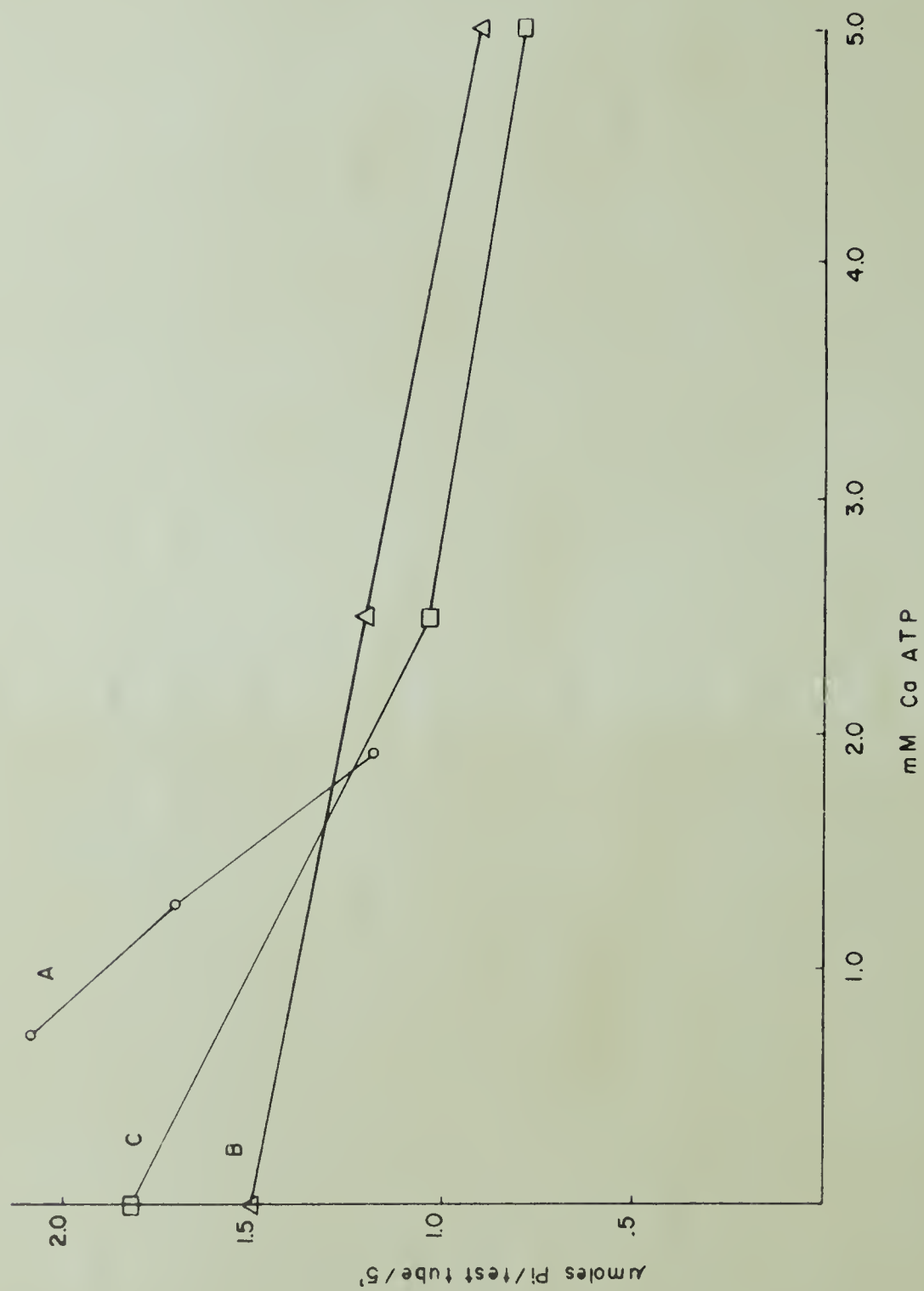
Plots of Ca ATP and free Ca^{++} concentrations against enzyme activity at different ATP concentrations are shown in Figures 20 and 21. These data show that at all ATP levels, the enzyme activity decreases with the Ca ATP concentration, while such a relation exists for free Ca^{++} only at 2.5 mM ATP (curve A, Figure 21). This latter observation can be explained by the fact that this is the only concentration of ATP (2.5 mM) at which free Ca^{++} reaches a significant level, and changes in its (Ca^{++}) concentration parallel changes in Ca ATP concentration. However, the slope of curve A, Figure 20 is greater than the slopes of either curve B or C in the same figure, indicating greater inhibition at 2.5 mM ATP. Therefore, free Ca^{++} may have some inhibitory effect in addition to that of Ca ATP and the reason for the steeper slope may be due to the additive inhibitory effects of Ca^{++} and Ca ATP.

It can be suggested that the CaATP complex competes with the enzyme. However, the depression of activity is not as large as it might be by a mere displacement of the substrate since Ca ATP itself can be hydrolyzed (see discussion).

Figure 20. Calculated CaATP concentration and ATPase activity in the presence of 5 mM $MgCl_2$ and different ATP levels. Taken from Table X . Curve A, 5.0 mM ATP; curve B, 10.0 mM ATP; curve C, 20 mM ATP. P_i determinations by LI method, 50 mM histidine, pH 7.2 (N=4).

Figure 21. Calculated free Ca^{++} concentrations and ATPase activity in the presence of 5 mM $MgCl_2$ and different ATP levels. Same as Figure 20 (N=4).

F. Effects of Zn^{++}

Because of the above mentioned relationship between Ca^{++}, Mg^{++} and the ATPase, and previous unpublished results from this laboratory (Daniel and Hutchinson) that chelators of Zn^{++} may inhibit ouabain induced contractures in the uterus, the effect of this cation on the microsomal ATPase activity was determined (Table XI).

TABLE XI

	ATP	mM Mg	Zn	μmoles P_i/mg.N/5' Activity
A	5	5	-	77.4
B	5	-	.5	17.1
C	5	-	5	29.7
D	5	5	.5	68.4
E	5	5	5	44.1

Table XI. Effects of Zn^{++} on fresh microsomal ATPase. Components were added as indicated. Aliquots were taken at 5 minutes for P_i determination by FS method. 50 mM Tris buffer, pH 7.2 (N=3).

FIGURE 22
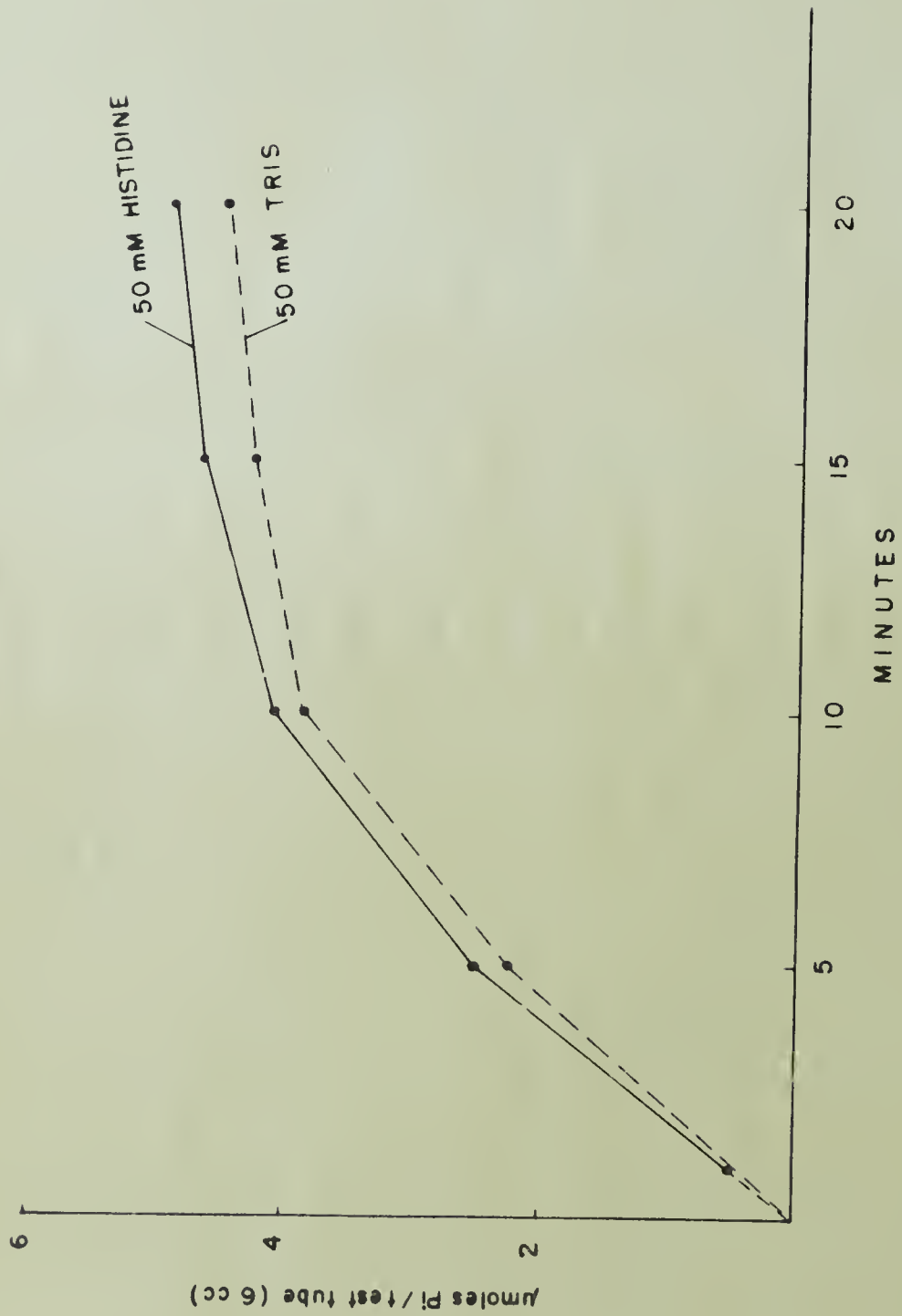
These experiments show that Zn^{++} as well as Mg^{++} and Ca^{++} can also cause some activation of the enzyme. Like Ca^{++}, when present along with Mg^{++}, Zn^{++} also causes a decrease in the activation (see discussion). Further studies related to Zn^{++}/ATP ratio were not carried out.

G. ADP Inhibition of Microsomal ATPase

Figure 22 shows that the rate of ATP hydrolysis by micro-somes decreases with time. High concentrations of ATP alone or Mg ATP, or of course low Mg ATP, decrease the enzyme activity (see section VA). Of the above, only low concentrations of Mg ATP could readily explain a decreased rate of hydrolysis with time. However, the fast initial rate of hydrolysis and subsequent decreased rate of several substrates suggested that product inhibition might also contribute to this effect.

The first possibility tested was the inhibition of the microsomal enzyme by ADP. ADP itself is hydrolyzed (about 45%) as shown in Table XII and acting as a substrate, requires a divalent cation. It can therefore be assumed that a MgADP complex is the actual substrate. Table XII also shows inhibition of ATP hydrolysis by ADP.

Figure 22. Activity time curve of fresh microsomal ATPase activity. Total volume was 6 cc., containing 30 μmoles $MgCl_2$ and 30 μmoles ATP (5 mM). One cc. aliquots were taken at times indicated. 50 mM histidine buffer, pH 7.2, top curve (N=4). 50 mM Tris buffer, pH 7.2, bottom curve (N=6). P_i determination by FS and LI methods.

TABLE XII

ATP	mM ADP	$MgCl_2$	μmole P_i/mg.N/5'
5	-	5	60.9
-	5	5	27.4
5	1.0	5	50.7
5	2.5	5	46.1
5	5	5	37.8

Table XII. Effects of ADP on fresh microsomal ATPase. Components were added as indicated. Aliquots were taken at 5 minutes for P_i determination by FS method. 50 mM Tris buffer, pH 7.2 (N=3).

Assay media were prepared with 2.5, 5.0, 10 and 20 mM ATP, all with 5 mM $MgCl_2$. Aliquots were taken at 5 and 10 minutes, and at 30 minutes, 5 mM ATP was added (in each case a .1 cc. volume of solution containing 15 μmoles of ATP was added to the remaining 3 cc. of assay medium Thus 15 μmoles was added to 3.1 cc., roughly a 5 mM concentration). After the addition of ATP, two more aliquots were withdrawn, one immediately after ATP addition, and one 10 minutes later. If substrate depletion was the only cause if the decreased slope of the enzyme activity-time curve, then addition of more ATP to form more Mg ATP should increase the activity.

FIGURE 23
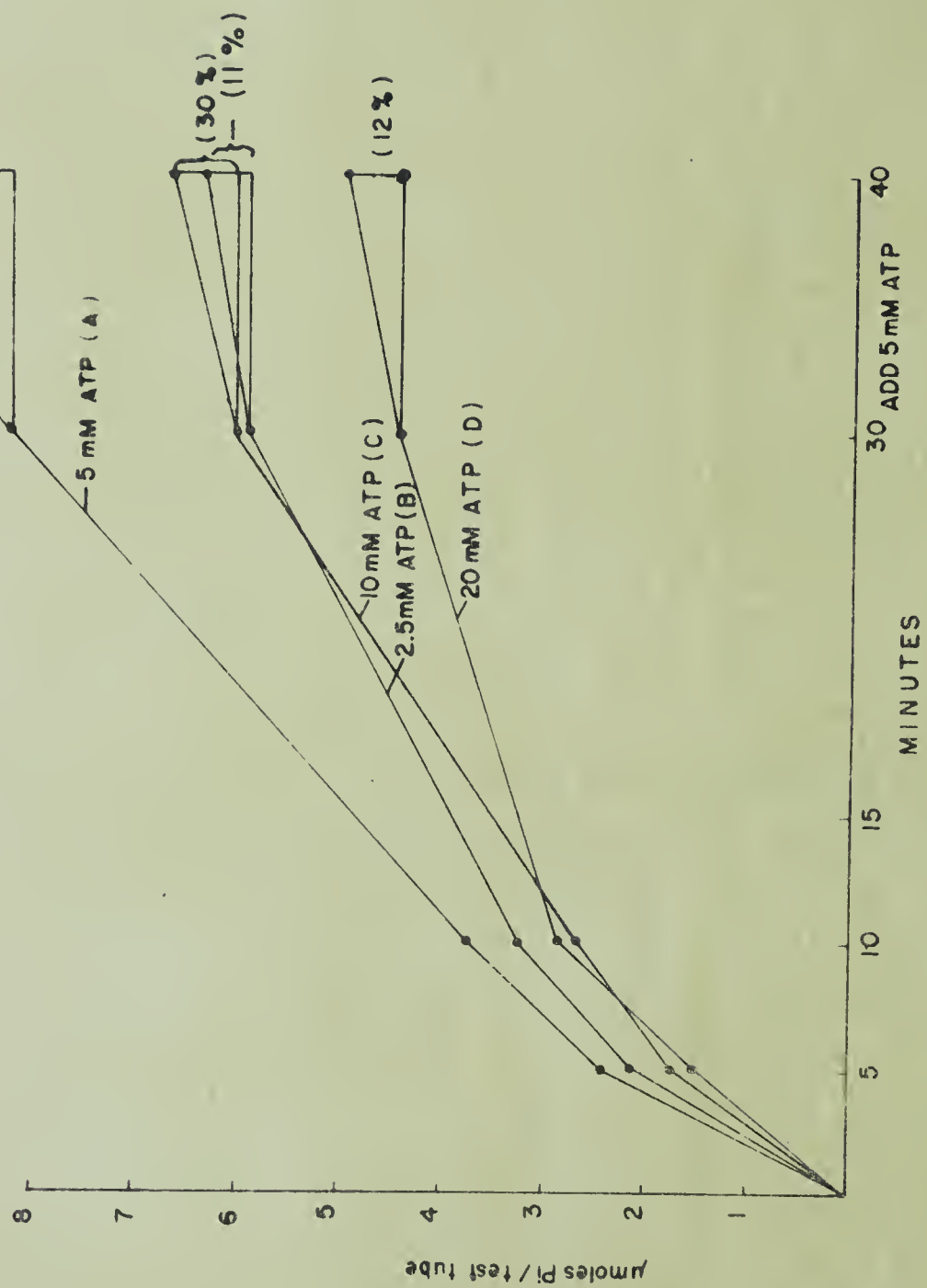
The results are shown in Figure 23.

At 30 minutes, the amount of ATP, Mg^{++} and ADP in 3.1 cc. after the addition of ATP assuming 15% hydrolysis of ADP, and residual ATP as calculated, was as follows:

	A	B	C	D
(μmoles)				
ATP	19.05	24.85	41.07	72.10
ADP	2.94	4.59	3.09	2.85
Mg	15.00	15.00	15.00	15.00

(15% ADP hydrolysis is assumed because its rate of hydrolysis is about 50% that of ATP (Table VIII) and 30% of the ATP was hydrolyzed in these experiments after 30 minutes)

Taking 100% in each experiment (i.e. A, B, C, D) as the amount of P_i detectable at the end of the first 10 minutes, the hydrolysis in the 10 minutes following addition of more ATP can be expressed as the percentage of the ATP hydrolysis in the first 10 minutes.

Figure 23. Addition of 5 mM ATP to fresh microsomal preparation at time approaching equilibrium. $MgCl_2$ concentration was always 5 mM. Aliquots were taken at 5, 10 and 30 minutes. After the 30 minute aliquot was taken, a .1 cc. volume containing 15 μmoles of ATP was added to each tube, and another aliquot taken. 50 mM Tris buffer, pH 7.2 P_i determination by FS method (N=3).

These percentages were:

A	B	C	D
95%	11%	30%	12%

The low value of D can be explained by the large excess of ATP relative to Mg^{++}. The low value of B can be explained by the relatively high concentration of ADP (4.6 μmoles/3 cc. = 1.5 mM). See Table XI. The intermediate value of C probably results from the excess of ATP (Mg^{++}/ATP ratio $\sim$1/3) and from the amount of ADP present, neither of which are as great as in D or B. The high value of A is due to the near optimal Mg^{++}/ATP ratio of 1/1.27.

The addition of ADP to a system with ATP present therefore inhibits the ATPase activity, even though ADP itself is hydrolyzed at about 45% of the ATP rate. The total ATP (at 5 mM) in 3 cc. assay medium is 15 μmoles. 2.5 mM ADP provides 7.5 μmoles in the same volume. If 20% of the ATP is hydrolyzed after 5 minutes, this will lead to the formation of 3.0 μmoles of ADP, or a concentration of 1.0 mM. This concentration of ADP inhibits the enzyme when added to the assay medium (Table XII). Furthermore, since the addition of P_i to the medium causes no inhibition (after correcting for P_i added), ADP contri-

butes to the decrease of the activity-time curve of the
enzyme. Whether this can explain all the decrease remains
undetermined.

It appears then, that at high ATP concentrations,
excess of ATP relative to Mg^{++}, or high concentrations of
Mg ATP itself, cause inhibition of activity, and that at
low ATP levels, substrate depletion as well as ADP accumu-
lation cause inhibition.

H. Summary

In the microsomal fraction of rat myometrium there is
an ATPase activity which can be activated by divalent cat-
ions, in the order $Mg > Ca \gg Zn$. The optimal Me^{++}/ATP ratio
is 1/1, determined only for Mg^{++} and Ca^{++}.

Ca^{++} inhibits the Mg ATPase activity. It competes
with Mg^{++} for ATP forming Ca ATP and lowering the Mg ATP
concentration. The resulting Ca ATP complex, while itself
being less hydrolyzed by the preparation than Mg ATP,
might be competitive with Mg ATP for the active site, and
inhibition becomes evident because of displacement of
Mg ATP by Ca ATP, and the lower hydrolysis of the latter.

The microsomal and mitochondrial preparations also
hydrolyze CTP, GTP, UTP and ITP, and para-nitrophenyl-
phosphate very slightly.

ADP is also hydrolyzed, but to a lesser extent

than ATP (about 50%). ADP can also inhibit enzyme activity, as can excess ATP and excess Mg ATP, as well as excess Mg^{++}.

The formation of ADP as the ATP hydrolysis progresses, and substrate depletion, are both responsible for the decrease in the enzyme-activity time curve.

VI. <u>Procedures Altering Microsomal ATPase Activity</u>

A. <u>Lack of Effect of Na^+ and K^+ on Fresh Fraction</u>

In subcellular fractions containing membrane fragments isolated from many tissues, an ATPase activity can be demonstrated which requires Mg^{++} and is activated by Na^+ and K^+ (see introduction). It has been suggested that this enzyme activity is involved in the active transport process.

Studies of the basic ATPase activity requiring Mg^{++} for activation were reported in the previous section (III). This section will deal with attempts to demonstrate Na^+ and K^+ activation of the Mg ATPase and with the effects of various compounds and treatments on the ATPase activity.

The first attempts at demonstrating univalent cation effects in the presence of Tris buffer gave the results presented in Table XIII. All fractions were studied.

TABLE X III

Fraction	Mg/ATP	μmoles P_i/mg N/5'			
		5Mg	5Mg+100Na	5Mg+20K	5Mg+100Na+20K
Nuclear	1/1	8.2	8.1	8.0	8.0
Mitochondrial	1/1	20.6	17.8	19.7	17.1
Microsomal	1/2	62.7	58.3	62.8	63.0
	1/1	80.7	71.8	81.2	72.6
	2/1	63.8	61.4	63.3	64.5

Table XIII. Effects of ions on ATPase of fresh subcellular
fractions. ATP concentrations were held constant at
5 mM, and the $MgCl_2$ was altered to achieve the ratios
indicated. Aliquots were withdrawn at 5 minutes for
P_i determination by FS method. 50 mM Tris buffer, pH
7.2 (N=4). Concentrations in mM.

Thus preliminary results, using only one concentra-
tion of both NaCl and KCl, failed to demonstrate any
marked univalent cation effects.

The above experimental design was obviously defi-
cient. The major drawback derives from the observations
of Skou (1960) that Na^+ in high concentration could in-
hibit the K^+ activation of the enzyme, and that high K^+
concentrations inhibited Na^+ activation. He assumed the
existence of 2 separate sites for cation activation on the

enzyme, one activated by Na^+ and with a higher affinity for Na^+ than for K^+, and the other activated by K^+, and with the order of affinities reversed. Apparently higher concentrations of either cation led to appreciable binding to the site for which it had lower affinity, thereby causing inhibition.

Thus an experiment testing lower concentrations of Na^+ and K^+ was tried. The results are presented in Table XIV.

TABLE XIV

| mM | | μmoles P_i/mg. N/5' | | Tris | | Histidine | |
Na^+	K^+	Mitochondrial	Microsomal			Mitochondrial	Microsomal
.5	.2	21.5	84.7			22.6	87.4
.5	2	22.0	85.3			23.8	86.9
.5	20	21.0	84.9			23.0	87.8
5	.2	19.7	83.8			22.4	87.1
5	2	20.8	84.7			23.3	87.9
5	20	20.4	84.1			23.6	88.0
50	.2	18.4	78.2			22.8	87.4
50	2	18.4	79.6			23.0	88.3
50	20	18.8	74.4			22.9	86.9
0	0	21.0	86.8			23.4	87.8

Table XIV. Effects of altered ion concentration on fresh mitochondrial and microsomal ATPases. [ATP] = [$MgCl_2$] = 5 mM. 50 mM Tris and histidine buffer, pH 7.2. Aliquots were withdrawn at 5 minutes for P_i determinations by the FS method (N=5).

FIGURE 24
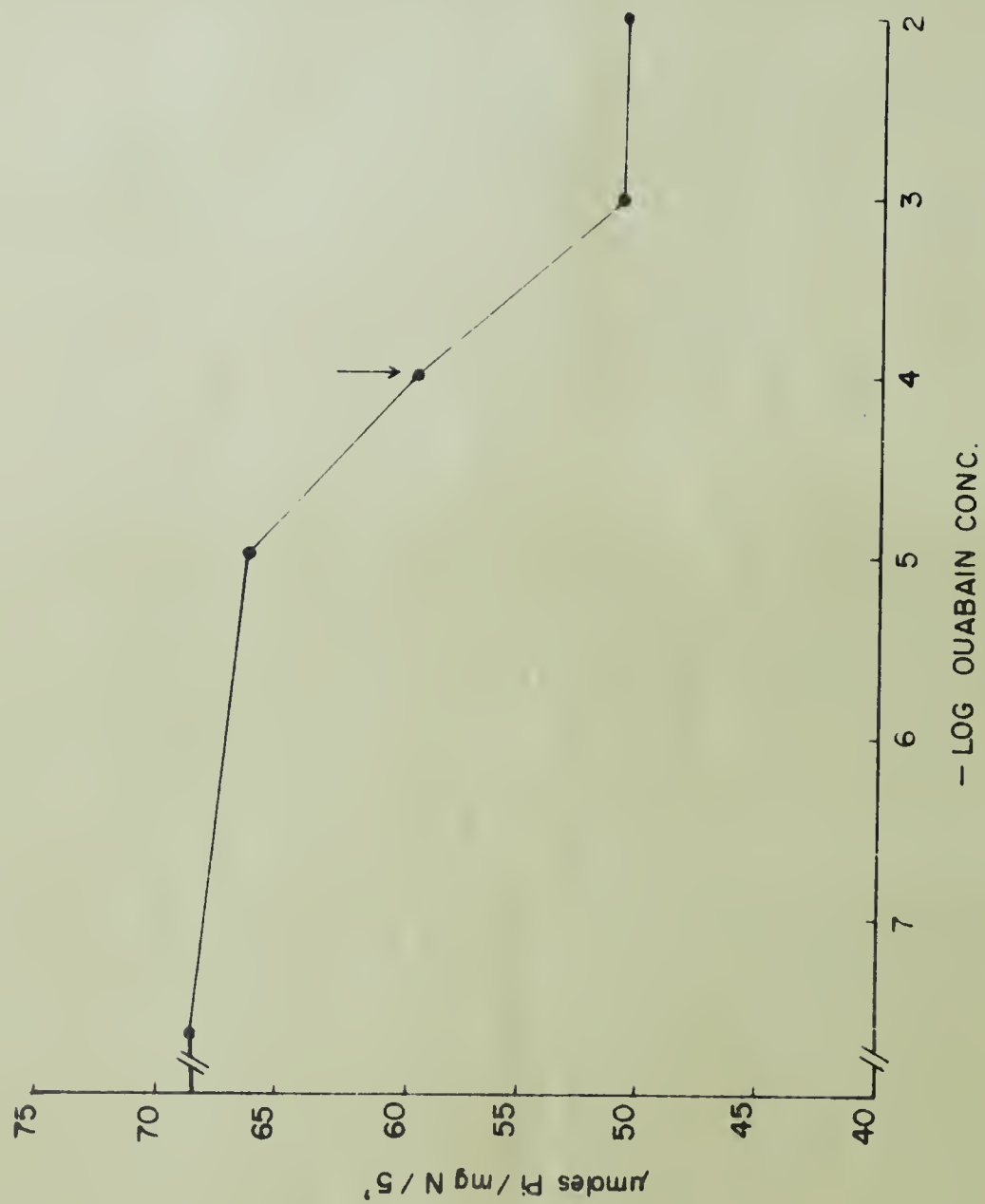
The data show that Na^+ in increasing concentrations
causes slight inhibition of both the mitochondrial and micro-
somal ATPases. K^+ was apparently without a definite effect.
The substitution of sucrose for Na^+ did not cause inhibition,
so the Na^+ effect was not an osmotic one, but due to the cat-
ion. Thus Na^+ inhibition, but no Na^+, K^+ or $Na^+ + K^+$ stimu-
lation was demonstrable in fresh material using Tris.

1) Effects of Ouabain and other Glycosides

The results in an earlier section with total homogenate
demonstrate that the overall ATPase activity requires about
10^{-3} M ouabain for maximal inhibition. This is considerably
higher than that required for most other tissues, but consis-
tent with the concentration required to produce maximal rates
of downhill ion movements (Daniel, 1963). This concentration
of ouabain was tried on the ATPase activity of the microsomal
fractions. The results shown in Table XV and Figure 24 indi-
cate that 1 mM ouabain inhibits the enzyme activity in the
presence of Na^+ and K^+ even if the cations do not stimulate
the activity, and in the absence of any monovalent cations.
The ouabain inhibition in each case is compared to the column
immediately preceding (e.g. A-B), and is expressed as an ab-
solute difference in activity.

Figure 24. Ouabain inhibition curve on fresh microsomal
    ATPase. Assay done in 50 mM histidine, pH 7.2 [MgCl$_2$]=
    [ATP]=5 mM. Ouabain added as indicated. Aliquots with-
    drawn at 5 min. for P_i determinations by LI method. Arrow
    indicates half maximal inhibition. (N=3)

TABLE XV

A)

	A	B	C	D	E	F	G	H
5 mM ATP	+	+	+	+	+	+	+	+
5 mM $MgCl_2$	+	+	+	+	+	+	+	+
100 mM NaCl	−	−	+	+	−	−	+	+
20 mM KCl	−	−	−	−	+	+	+	+
1 mM ouabain	−	+	−	+	−	+	−	+
	68.2	59.3	66.7	59.7	68.0	58.1	61.7	54.7
Inhibition		A−B 8.9		C−D 7.0		E−F 9.9		G−H 4.0

B)

	A	B	C	D	E	F	G	H
5 mM ATP	+	+	+	+	+	+	+	+
5 mM $MgCl_2$	+	+	+	+	+	+	+	+
200 mM NaCl	−	−	+	+	−	−	+	+
200 mM KCl	−	−	−	−	+	+	+	+
1 mM ouabain	−	+	−	+	−	+	−	+
	81.4	72.5	74.0	70.8	80.6	69.7	65.6	64.7
Inhibition		A−B 8.9		C−D 3.2		E−F 10.9		G−H 0.9

Table XV. Effects of 10^{-3} M ouabain on fresh microsomal ATPase (Tris buffer). (+) means compound in left column was added, (−) means it was absent from the assay medium. Differences are as indicated in the text. Aliquots were withdrawn at 5 minutes for P_i determinations by FS method.

The use of inactive glycosides and other active ones in the assay showed that the inhibitory effect, small as it may be, was specific. 1 mM hexahydroscillaren, an inactive preparation (a gift from Dr. Arnold Schwartz), did not inhibit the ATPase activity at all, while Scillaren, an active preparation (Sandoz), had effects similar to ouabain in concentrations of .01-1 mg./cc. assay medium. (Molecular weight cannot be determined since this preparation is a mixture of active glycosides.) Increasing the ouabain concentration to 10^{-2} M did not cause further inhibition, and increasing Na^+ or K^+ concentrations to 200 mM, did not overcome the ouabain inhibition. However, increasing both Na^+ and K^+ each to 200 mM together did have an effect on the ouabain inhibition (G-H, Table XVB). Ouabain did not further inhibit this value, which was already very low compared to column A. This may indicate that Na^+ and K^+ antagonize the action of ouabain. Since experiments with osmotic substitutes were not performed, the question of specific or osmotic effects of these high ionic concentrations causing ATPase inhibition and antagonizing ouabain cannot be answered.

Figure 24 shows the extent of ouabain inhibition in the series of experiments which gave maximum inhibition. The actual range of inhibition in the presence of ATP and Mg^{++} was 5% to 30%. The extent of ouabain inhibition does not appear to be related to the presence of Na^+ or Na^+ activation (Table XVA).

a. $\underline{K^+ \text{ effects}}$. Table XVI shows the inability of increasing concentrations of K^+ to overcome the ouabain inhibition of fresh microsomal ATPase.

TABLE XVI

mM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
5 ATP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5 Mg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100 Na	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
K	-	-	-	-	20	20	20	20	50	50	50	50	100	100	100	100
1 Ouab.	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
	86.1	71.5	86.5	71.4	84.4	72.5	83.6	72.0	81.4	73.5	81.4	70.0	82.6	72.1	83.1	72.5

Table XVI. Inability of increasing concentrations of K^+ to overcome the ouabain inhibition of fresh microsomal ATPase. Aliquots were taken at 5' for P_i determinations by LI method. + means the compound in the left column was present in the assay, and - means it was absent. Enzyme activity expressed in μmoles P_i/mg. $N/5'$ (N=5).

2) <u>Tris vs. Histidine Buffer</u>

All the above studies with Na$^+$ and K$^+$ were done in the presence of 50 mM Tris buffer. The same studies were repeated in 50 mM histidine buffer and the results are given in Tables XVII and XVIII. The most important difference

TABLE XVII

	A	B	C	D
Microsomal	5 Mg	5Mg+100Na	5Mg+20K	5Mg+100Na+20K
Histidine	73.8	73.4	72.7	74.1
Tris	68.2	66.7	68.1	66.4

Table XVII. Effects of different buffers on the cation effects on fresh microsomal ATPase. Assays were done in the usual manner with $[MgCl_2]=[ATP]=5$ mM. The total homogenate was divided into 2 halves. Both halves were centrifuged as usual, and one resulting microsomal fraction was suspended in 50 mM Tris, and the other in 50 mM histidine. P_i determinations were done by LI method (N=4) (The results were similar with mitochondrial ATPase)

TABLE XVIII

	A	B	C	D	E	F	G	H
5 mM ATP	+	+	+	+	+	+	+	+
5 mM MgCl$_2$	+	+	+	+	+	+	+	+
100 mM NaCl	–	–	+	+	–	–	+	+
20 mM KCl	–	–	–	–	+	+	+	+
1 mM ouabain	–	+	–	+	–	+	–	+
	79.3	70.4	79.4	69.7	78.9	70.3	79.7	71.8
Inhibition		A-B 8.9		C-D 9.7		E-F 8.6		G-H 7.9

Table XVIII. Effects of 10^{-3} M ouabain on fresh microsomal
   ATPase (histidine buffer). Same as Table XV   but using
   50 mM histidine instead of Tris.

---

from the results in Tris buffer was the fact that while Na^+

did not stimulate in histidine, it did not inhibit the enzyme

(Table XVII, and compare columns A and B of Tables XV   and

XVII). The results of Table XV   were not all from the same

experiment (i.e. Table XVA was a different experiment than

XVB).   A direct comparison of Tables XV and XVIII shows that

regardless of the buffers used, ouabain inhibition was un-

related to Na^+ presence in fresh microsomal fractions.

   Since experiments comparing Tris and histidine buffers

using only ATP and Mg^{++} (Table XVII column A) shows that

histidine confers slightly higher activity on the enzyme,

lack of Na^+ inhibition in this buffer could not be due to

histidine itself inhibiting the enzyme. In fact, histidine

slightly stimulates the enzyme, which could be the reason

for lack of Na^+ inhibition.

   B.   Treatments Giving Na^+ Stimulation

   1) DOC Treatment

   Skou (1962) showed that the presence of the detergent,

DOC, during the isolation of kidney and brain microsomes

increased Na^+ and K^+ stimulation of the resultant enzyme.

FIGURE 25
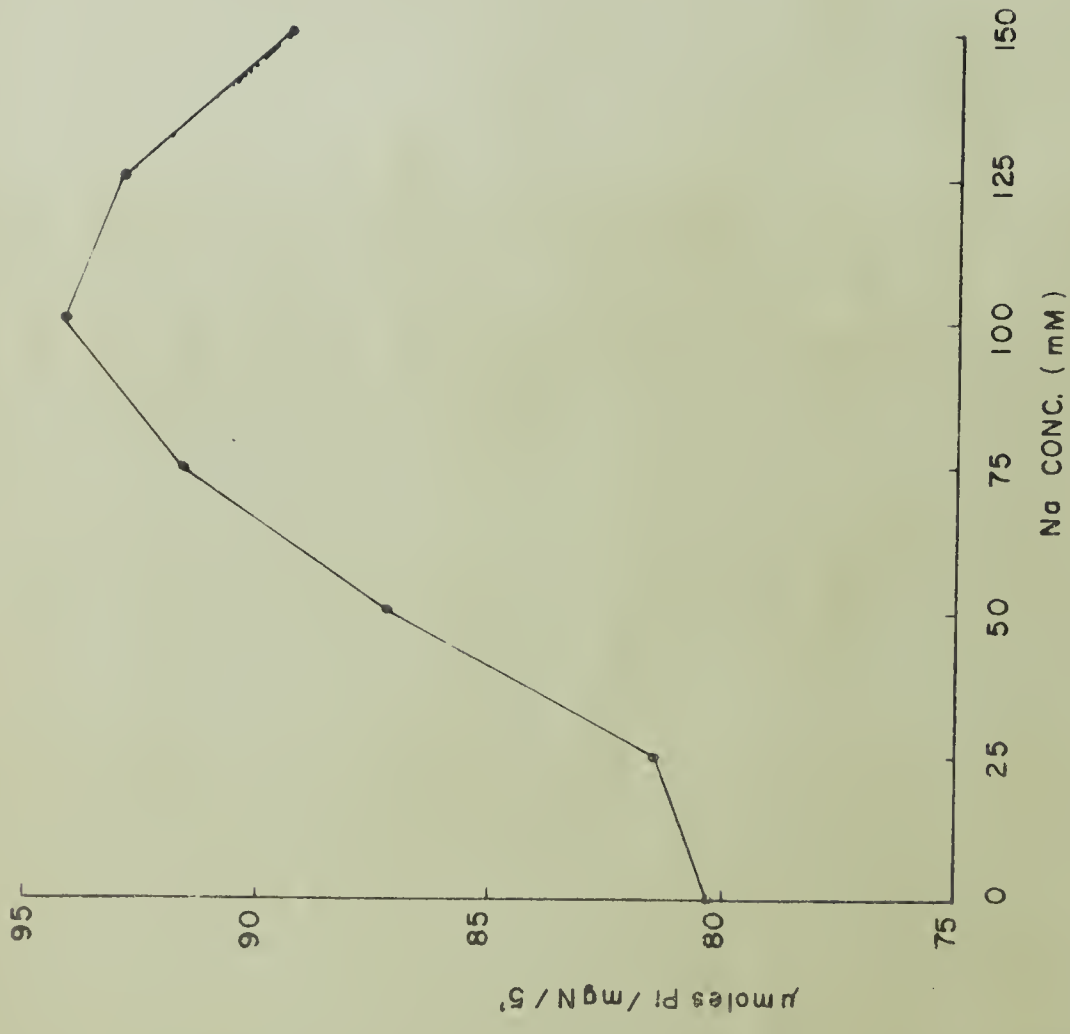
Since the fresh microsomal fraction of rat myometrium showed no cationic stimulation, various concentrations of DOC were added at various stages of the isolation procedure to determine whether Na^+ or $Na^+ + K^+$ stimulation could be produced.

Method 1.

The uteri were excised, homogenized and centrifuged as usual. The isolating media had sucrose, and one of the following concentrations of Na DOC.

%	mM
.041	1
.100	2.3
.300	6.9

The fractions collected were then suspended in either Tris or histidine. Regular assays were then performed, and these results are presented in Table XIX.

After this procedure, no stimulation by Na^+ or $Na^+ + K^+$ was evident in Tris buffer. However, with histidine buffer, slight Na^+ stimulation was seen with .1% DOC, with no further increase upon addition of K^+.

Further examination of the .1% DOC isolated microsomal fraction showed that increasing the Na^+ over 100 mM failed to stimulate, as did the use of less Na^+ (Figure 25).

Figure 25. Effects of Na^+ on DOC isolated ATPase activity. Each assay contained 5 mM $MgCl_2$, 5 mM ATP and the designated amount of Na^+. 50 mM histidine buffer, pH 7.2 P_i determination with FS and LI methods (N=4).

TABLE XIX

μmoles P_i/mg.N/5'

DOC (%)	50 mM histidine									50 mM Tris								
	.041%			.1%			.3%			.041%			.1%			.3%		
Ionic Media	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Nuclear	7.4	7.7	7.8	-	-	-	-	-	-	7.5	7.3	7.0	-	-	-	-	-	-
Mitochondrial	19.4	18.0	18.4	10.7	10.8	11.7	-	-	-	20.6	17.1	18.0	11.7	10.0	10.4	-	-	-
Microsomal	90.8	91.4	92.3	82.1	92.1	92.0	15	16.3	17	84.8	80.9	84.4	78.1	80.1	80.1	14.3	17.0	14.0

Table XIX. Effects of isolation in DOC (Method 1) on microsomal ATPase. Assay 1, 5 mM ATP, 5 mM MgCl$_2$; assay 2, 5 mM ATP, 5 mM MgCl$_2$, 100 mM NaCl$_2$; assay 3, 5 mM ATP, 5 mM MgCl$_2$, 100 mM NaCl, 20 mM KCl. P_i determinations by FS method. 50 mM Tris buffer, pH 7.2, as indicated.

In none of these DOC treated fractions could any K^+ stimulation be demonstrated.

Method 2.

The microsomal fraction itself was treated with DOC. This was done in two ways;

a) by adding DOC to the assay medium as well as the isolation medium, and

b) treating the microsomal suspension with DOC, recentrifuging the pellet, and then resuspending. The results are presented in Table XX and were performed in histidine only. Slight Na^+ stimulation was demonstrable in procedure (b) at a DOC concentration of 0.1%. No K^+ activation was observed.

TABLE XX

	a mM			b mM		
% DOC	5 Mg	5Mg,100Na	5Mg,100Na,20K	5 Mg	5Mg,100Na	5Mg,100Na,20K
.041	75.7	76.1	76.2	76.4	78.2	78.1
.100	53.2	52.9	51.4	49.8	56.1	56.2
.300	21.8	22.7	22.8	40.3	41.4	40.7

Table XX. Effects of different procedures on Na^+ stimulation of ATPase by DOC. The 2 procedures are explained in the text. Each procedure was tried at the 3 indicated concentrations of DOC, and the cation effects tested. All concentrations in mM. [ATP] = 5 mM. 50 mM histidine buffer, pH 7.2. P_i determined by FS method.

The substitution of Na lauryl sulfate for DOC at .040%, .1% and .3% gave no Na^+ or $Na^+ + K^+$ stimulation in similar experimental procedures (i.e. in both methods 1 and 2).

Na^+ stimulation will be discussed subsequently in terms of the activity ratio, defined as follows:

Actvity Ratio

$$= \frac{\text{Enzyme activity with } Mg^{++} + Na^+}{\text{Enzyme activity with } Mg^{++}}$$

The maximum value of the activity ratio obtained with DOC was 1.18 (i.e. the maximum Na^+ stimulation was 18%).

2) Storage at $-5^{\circ}C$

Working with ATPase from cardiac tissue, Schwartz (1962) was unable to demonstrate any $Na^+ + K^+$ stimulation of the enzyme until storage of the tissue at $-5^{\circ}C$. The effects of similar ageing of the microsomal and mitochondrial fractions of the rat myometrium were studied.

Following isolation of these two fractions in the usual manner, the pellets were suspended in either 50 mM Tris or 50 mM histidine, both at pH 7.2. An aliquot of these suspensions was assayed for ATPase activity in the standard manner. The remainder of each suspension was

divided up into small aliquots (~ 1 cc.) and placed in a

walk-in freezer at -5°C. At various times after isolation

a sample was thawed at room temperature, and assayed for

ATPase activity. Some of the results are seen in Table

XXI.

TABLE XXI

mM		μmoles P_i/mg.N/5' Activity	
Na	K	Mitochondrial	Microsomal
.5	.2	13.4	64.9
.5	2	14.6	64.1
.5	20	15.3	65.6
5	.2	14.7	65.1
5	2	14.9	65.3
5	20	16.8	64.9
50	.2	14.7	72.1
50	20	15.2	72.0
100	.2	16.3	96.0
100	2	14.7	98.0
100	20	14.0	97.4
0	0	14.7	64.1

Table XXI. Na^+ and K^+ effects on stored microsomal
   ATPase (1 week). [ATP]=[MgCl$_2$]=5 mM. 50 mM histi-
   dine buffer,pH 7.2 (N=10).

In no case was any further increase in activity seen by adding K^+ to the already increased activity due to the addition of Na^+. The hydrolysis of ADP, AMP, CTP, GTP, ITP or UTP was not stimulated by Na^+ (Table XXII).[*]

TABLE XXII

μmoles P_i/mg. N/5'

	5 mM MgCl$_2$	5 mM MgCl$_2$+100 mM Na	Activity Ratio
ATP	31.6	51.6	1.6
ADP	17.4	17.2	1.0
AMP	4.3	4.0	1.0
CTP	32.7	33.0	1.0
GTP	36.5	34.0	1.0
ITP	40.1	40.9	1.0
UTP	30.7	32.1	1.04

Table XXII. Effects of Na^+ on hydrolysis of various nucleotides by stored microsomal fractions. All nucleotide concentrations were 5 mM. 50 mM histidine buffer, pH 7.2 (N=4). [MgCl$_2$] = 5 mM.

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a. $\underline{K_M}$ value of Na^+

The K_M value of Na^+, found graphically from the data of Table XXI, was 20 mM. This is approximately 5-10 times the K_M values found for other tissue enzyme preparations (.4-1.4 mM, from Skou, 1965).

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[*] This is further indication that the ADPase activity is not due to adenylate kinase since if the ATPase were responsible for the release of P_i, one would expect Na^+ stimulation of the ADPase activity as well.

b. Effects of ouabain, other glycosides, and K^+

In all other tissues studied (see introduction),
the Na^+ + K^+ stimulation was abolished by the addition
of ouabain, and in the absence of Na^+ and K^+ stimulation,
ouabain had no effect. In the fresh microsomal fraction
of rat myometrium, the ouabain inhibitory effect was pre-
sent even though there was no univalent cationic stimula-
tion, in the absence or presence of added Na^+ or Na^+ and
K^+. The ouabain inhibition varied between 10% to 30% of
the activity in the presence of Mg^{++} + Na^+ in fresh micro-
somes. Table XXIII shows the effect of ouabain on the Na^+
activated portion of the ATPase activity of aged microsomes,
and summarizes the storage effects.

TABLE XXIII

	A	B	C	D		
	5 mM ATP 5 mM $MgCl_2$	5 mM ATP 5 mM $MgCl_2$ 100 mM NaCl μmoles P_i/mg N /5'	5 mM ATP 5 mM $MgCl_2$ 100 mM NaCl 20 mM KCl	5 mM ATP 5 mM $MgCl_2$ 100 mM NaCl 20 mM KCl 1 mM ouabain	B÷A	$\frac{B-D}{B}$x100
1) 0 day	75.5	76.2 (101%)	75.5	63.5 (84%)	1.01	16.6
7 day	43.5	65.9 (151%)	66.7	61.4 (140%)	1.51	08.8
2) 0 day	60.7	55.8 (92%)	54.7	48.5 (80%)	.92	13.0
7 day	31.6	51.6 (163%)	52.3	47.4 (153%)	1.63	08.1
12 day	31.3	46.2 (148%	46.3	--	1.48	-
3) 0 day	43.9	45.4 (104%)	44.6	38.6 (89%)	1.04	14.9
6 day	25.6	36.9 (144%)	36.5	28.5 (122%)	1.44	22.7
4)		Storage in 50 mM Tris, pH 7.2				
0 day	40.1	37.9 (95%)	36.9			
6 day	18.1	21.2 (117%)	21.7		.95	
					1.17	

Table XXIII. Effects of storage at $-5^{\circ}C$ on microsomal
ATPase activity. Assays were done in buffer indicated.
P_i determinations were done by the FS and LI methods.
If column A in each experiment is 100%, column B rep-
resents Na^+ stimulation ($>100\%$) and % ouabain inhibi-
tion is represented by $(\frac{B-D}{B})$ x 100.

Thus in the aged microsome fraction, Na^+ stimulated

the ATPase activity, and ouabain could partially inhibit

this activation.

The B $\div$ A column in Table XXIII is the activity ratio,

while the $(\frac{B-D}{B})$ x 100 column is the per cent ouabain inhibi-

tion. There was an increase in the activity ratio with age-

ing. The reason for this increase was that the Mg^{++} activity

decreased faster than the $Mg^{++} + Na^+$ activity. The possible

mechanisms will be discussed later.

A direct comparison of the activity ratio and the

ouabain inhibition reveals that the ouabain inhibition was

unrelated to the Na^+ stimulation except in #3.

Table XXIV shows the effects of 1 mM ouabain on the

ATPase activity of stored microsomal fractions. The glyco-

side could still slightly inhibit   the activity in the

absence of Na^+ or K^+ (about 11%), so not only the Na^+ stimu-

lated portion is affected. It seems that the conditions

which show the largest ouabain effects also show Na^+ stimu-

lation. It appears, then, that there may be some relation

TABLE XXIV

	A	B	C	D	E	F	G	H
5 mM ATP	+	+	+	+	+	+	+	+
5 mM MgCl$_2$	+	+	+	+	+	+	+	+
100 mM NaCl	-	-	+	+	-	-	+	+
20 mM KCl	-	-	-	-	+	+	+	+
1 mM ouabain	-	+	-	+	-	+	-	+
	47.9	42.4	67.4	56.2	47.3	41.4	67.9	56.9
			AR=1.41		AR<1.0		AR=1.42	
Inhibition		A-B	C-D		E-F		G-H	
		5.5	11.2		5.9		11.0	

Table XXIV. Effects of 10^{-3} M ouabain in stored microsomal ATPase. Same as Tables XIV and XII, except histidine buffer used throughout.

AR = activity ratio.

FIGURE 26
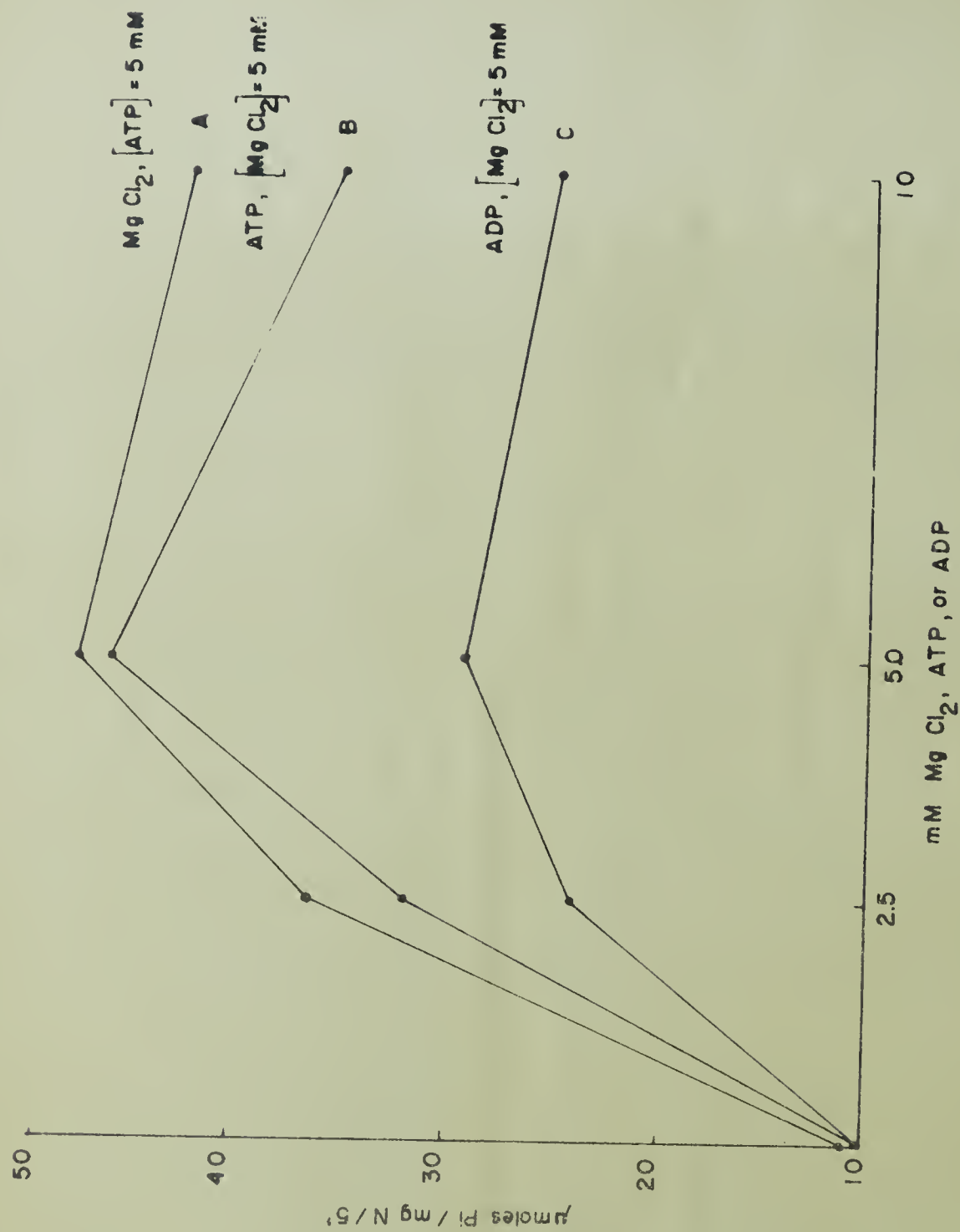
between Na^+ stimulation and ouabain inhibition (Table XXIII, experiment #3).

Table XXV shows the inability of increasing concentrations of K^+ to overcome the ouabain effect. It should also be noted that hexahydroscillaren in a concentration of 1 mM had no inhibitory effects while Scillaren (.01-.1 mg./cc. assay medium) had the same effect as ouabain on the stored microsomal fraction.

c. Alterations of Mg^{++}/ATP ratio

Figure 26 shows the effects of varying the Mg^{++}/ATP ratio on the ADPase and ATPase activities in the stored microsomal fractions in the absence of Na^+. As in fresh material, the optimum ratio was 1/1. A comparison of curves A and B of Figure 26 with Figure 10, and curve B of Figure 13 indicate that ageing did not alter the Mg^{++} effect on the enzyme. Studies similar to those of Figure 13, curve B, were not carried out on stored microsomal fractions.

Table XXVI shows the same results with and without Na^+ and K^+. In all cases the highest enzyme activity was recorded when the Mg^{++}/ATP ratio was 1/1. The activity ratio could not be increased over that obtained with a 1/1 Mg^{++}/ATP ratio. The lower activity ratio observed at Mg^{++}/ATP equal

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Figure 26. Optimal Mg^{++}/ATP ratio in stored microsomal fractions. Curve A, [ATP]=5 mM and $MgCl_2$ varied as indicated (N=3). Curve B, [$MgCl_2$]=5 mM, and ATP varied as indicated (N=3). Curve C, [$MgCl_2$] =5 mM, and ADP varied as indicated (N=2).

TABLE XXV

mM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
5 ATP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5 Mg	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100 Na	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
K	-	-	-	-	20	20	20	20	50	50	50	50	100	100	100	100
1 Ouab.	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
	46.2	34.3	62.4	42.1	46.8	34.2	63.3	40.3	44.5	33.3	60.8	41.0	43.1	32.8	53.8	39.4

Table XXV. Inability of increasing K^+ concentrations to overcome ouabain inhibition of ATPase activity of stored microsomes. + means the compound in the left column was present, - means it was absent. Aliquots taken at 5 minutes for P_i assay by LI method. Activity expressed as μmoles P_i/mg. N/5' (N=3).

TABLE XXVI

Mg(mM)	ATP(mM)	Na mM	K	μmoles P$_i$/mg.N/5'	Activity Ratio
2.5	5.0	0	0	37.5	
2.5	5.0	100	0	53.5	1.42
2.5	5.0	100	20	53.0	
5.0	5.0	0	0	48.6	
5.0	5.0	100	0	73.2	1.51
5.0	5.0	100	20	73.5	
10.0	5.0	0	0	43.5	
10.0	5.0	100	0	65.4	1.50
10.0	5.0	100	20	66.0	
5.0	2.5	0	0	32.5	
5.0	2.5	100	0	45.7	1.40
5.0	2.5	100	20	45.0	
5.0	5.0	0	0	47.1	
5.0	5.0	100	0	70.1	1.49
5.0	5.0	100	20	70.0	
5.0	10.0	0	0	30.0	
5.0	10.0	100	0	44.8	1.49
5.0	10.0	100	20	44.1	

Table XXVI. Mg^{++}/ATP ratio and Na$^+$ stimulation of ATPase in stored microsomes. Standard assay procedures, using both FS and LI for P$_i$ determinations. Components added as indicated. (N=3).

FIGURE 28

CURVE A — CURVE B

FIGURE 27

STORAGE IN 50 mM HISTIDINE

STORAGE IN 50 mM TRIS

WEEKS
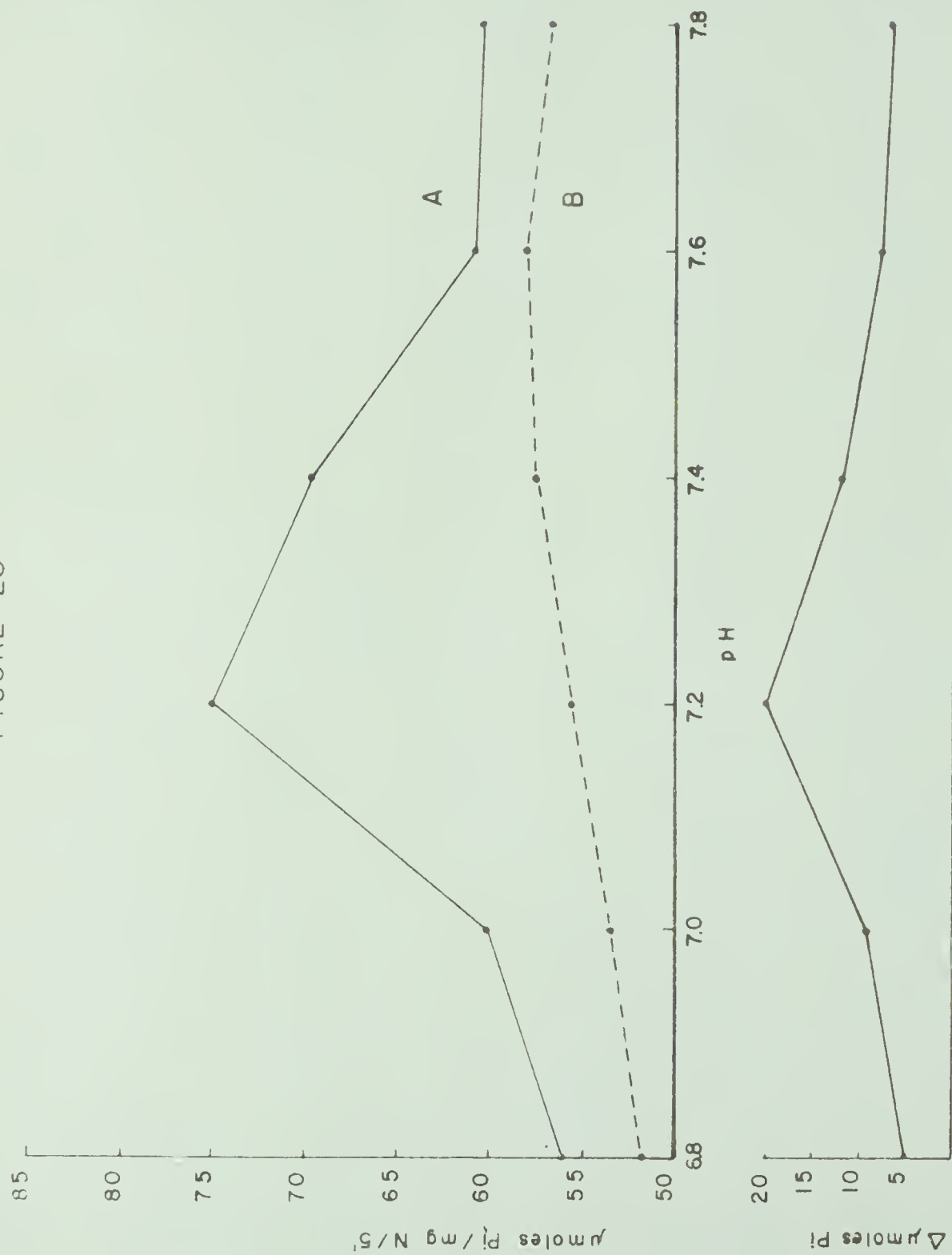
ACTIVITY RATIO (Mg+Na / Na)
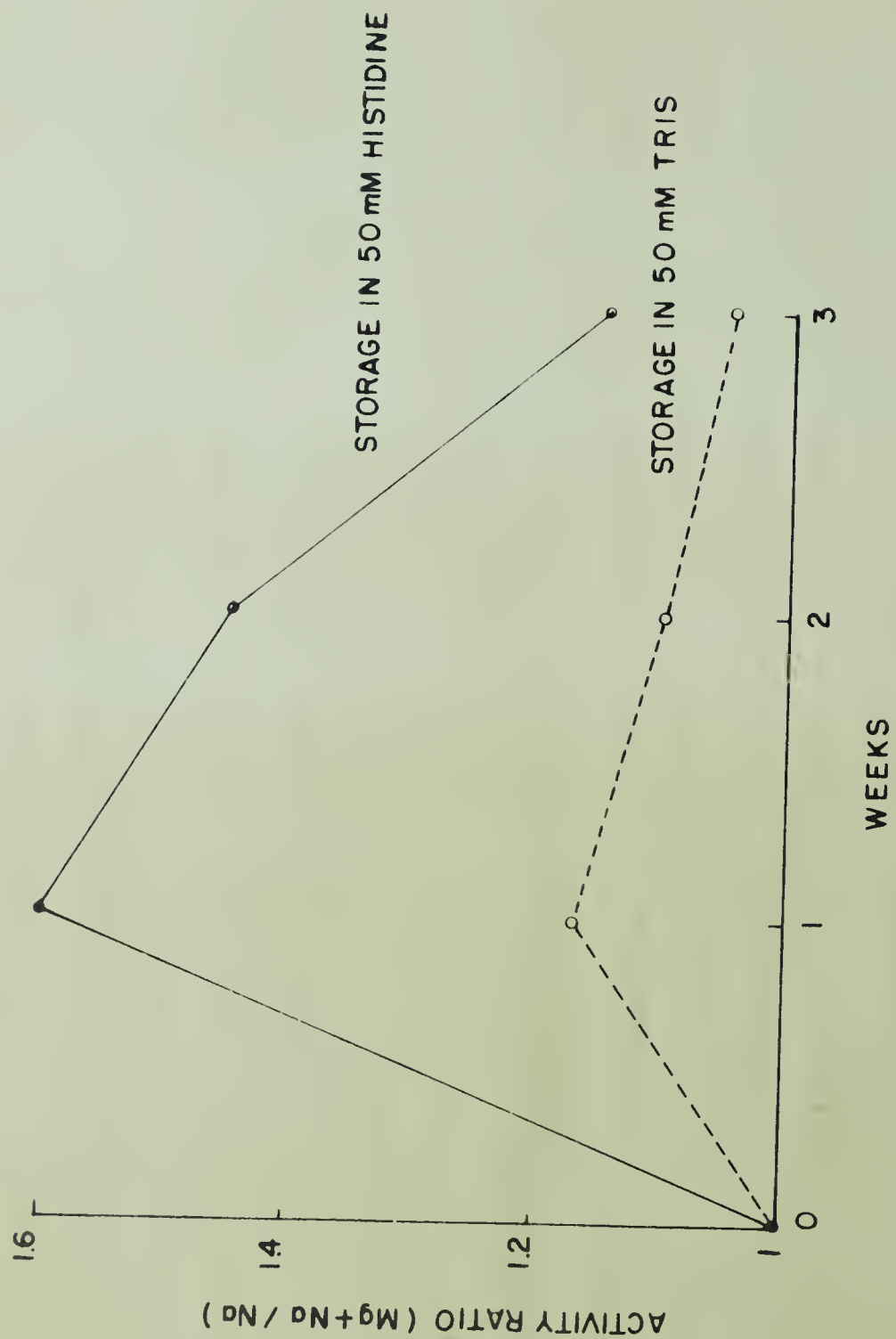
to 2.5/5.0 and 5.0/2.5 may result from low substrate (MgATP) concentrations.

In cardiac tissue, Schwartz (1962) showed that the activity ratio $(\frac{Mg^{++} + Na^+ + K^+}{Mg^{++}})$ increased with increasing storage time. This was not true for myometrium as can be seen from Figure 27, which also compares Tris and histidine storage effects.

In Table XXI (and in Tables XXIV-XXVII) no K^+ effect can be seen, while Na^+ stimulation of the ATPase activity is always present.

d. pH-activity curve of stored microsomes.

Figure 14 shows a very broad pH-activity curve of fresh microsomes. Figure 28 shows the pH activity curve of stored material. It can be seen that while the Mg^{++} stimulated ATPase activity still has a broad band, a peak occurs at about pH 7.2 with the Na^+ activated portion. Furthermore upon subtracting the Mg^{++} stimulated activity from the $Na^+ + Mg^{++}$ activity the resulting curve at the bottom of Figure 28 shows

Figure 27. Effect of cold storage on $Mg^{++}+Na^+/Mg^{++}$ (-5°C). Microsomes were stored and assayed in the specified buffer. Assay conditions are the same as in Table XIX, except both FS and LI methods were used for P_i determinations.

Figure 28. pH activity curve of stored microsomal ATPase activity. Assays were run with 5 mM Mg^{++} and with 5 mM Mg^{++} + 100 mM Na^+ at various pH values. Aliquots were taken at 5 minutes for P_i determination by LI method. (N=3). Curve A-curve B gives pH curve of Na^+ activated portion. Curve A is activity with 100 mM NaCl and 5 mM $MgCl_2$. Curve B is activity without NaCl.

that the Na^+ activated ATPase appears to have a peak at pH 7.2.

3) Combination of DOC and storage

Since both ageing and DOC treatment allow Na^+ stimulation, both treatments were combined to see if the activity ratio in the microsomal fraction could be increased. This was tested in 3 different ways.

	Isolation	Storage (1 week)
Control 1	0 DOC	no storage
Control 2	0 DOC	0 DOC
1	0 DOC	.1 DOC
2	.1% DOC	0 DOC
3	.1% DOC	.1% DOC
Control 3	.1% DOC	no storage

0 DOC indicates either that the usual procedure of isolation was used without any additions of the detergent, or that storage was done in 50 mM histidine, without DOC.

.1% DOC means that that amount of DOC had been added to either the isolation medium (.25 M sucrose) or to the storage medium (50 mM histidine).

TABLE XXVII

Procedure	5 Mg	5Mg,100Na	5Mg,100Na,20K	Activity Ratio
Control 1	84.7	85.3	85.3	1.01
Control 2	41.7	64.4		1.54
1	53.4	67.6	67.9	1.27
2	47.4	71.7	71.9	1.51
3	48.4	58.1	58.3	1.20
Control 3	43.7	53.4	34.4	1.22

Table XXVII. Effects of combination of storage and DOC treatment on the activity ratio of microsomal ATPase. Each procedure was tried separately on 4 separate occasions. Assays were done in 50 mM histidine, pH 7.2, [ATP]=5 mM, using the Li method for P_i determination. (N=3)

The results are presented in Table XXVII, and indicate that a combination of both procedures did not increase the Na^+ stimulation, or impart any K^+ dependence to the ATPase activity.

4) Urea Isolation

Another procedure which slightly increased the activity ratio was isolation of the microsomal fraction in 1.0 M urea (Table XXVIII). The activity ratio increase was approximately the same as that achieved with DOC.

TABLE XXVIII

Treatment	(A) 5 Mg	(B) 5Mg,100Na	μmoles P$_i$/mg.N/5' (C) 5Mg,100Na,20K	(D) 1mM ouabain 5Mg,100Na,20K	$\frac{B}{A}$	$\frac{(C-D)}{C}$ 100
Control	85.7	86.8	86.4	72.7	1.02	15.8%
1.0 M Urea	61.7	72.5	71.7	63.7	1.24	11.1%

Table XXVIII. Effects of urea on the activity ratio of fresh microsomal ATPase. 1.0 M urea was included in the entire isolation procedure, but not added to either the suspending or the assay medium. Assay procedure was carried out as usual in 50 mM histidine buffer, pH 7.2, using LI method for P$_i$ determination (N=3).

A combination of these 2 procedures did not increase Na^+ stimulation any further, nor was ouabain inhibition increased.

5) NaF Addition

Another procedure which altered the activity ratio was addition of NaF to the assay medium. The results of such an experiment are shown in Table XXIX.

TABLE XXIX

	ATP	MgCl$_2$	NaF	NaCl	μmoles P$_i$/mg.N/5'	Activity Ratios
A	5mM	5 mM	–	–	156.1	
B	5	5	100	–	37.0	
C	5	5	10	–	90.0	$\frac{G}{C} = 1.20$
D	5	5	1	–	136.8	
E	5	5		100mM	151.2	$\frac{H}{D} = 1.15$
F	5	5	100	100mM	32.4	
G	5	5	10	100mM	108.1	
H	5	5	1	100mM	156.6	

Table XXIX.   Effects of NaF on fresh microsomal ATPase activity. Components were added as indicated. P$_i$ determinations by LI method. 50 mM histidine, pH 7.2.

These results indicate that at concentrations of 10 mM and 1 mM, NaF gives some Na^+ stimulation. Comparable quantities of NaCl replacing NaF did not activate (see Table XXVIII, so it must be concluded that the F^- is exerting some effect on the system. Table XXIX also demonstrates the possibility that the results could be due to Cl^- antagonism to F^-.

In one experiment done to test the K^+ effect on the ATPase in the presence of NaF, there was no effect alone, or in the presence of Na^+.

A suggestion may also be made that the F^- molecule is complexing Mg^{++}. This mechanism would act by producing a suboptimal ATP/Mg ratio. The complexing theory as the mechanism of F^- inhibition may be excluded since, as shown in Table XXX, the addition of Mg^{++} did not overcome the NaF inhibition.

Increasing the Mg^{++} concentration at 100 mM NaF did partially overcome the NaF but this did not happen at 10 mM NaF. Instead, increasing Mg^{++} concentrations increased the inhibition of ATPase activity. The extent to which Mg^{++} inhibition of ATPase activity in the presence of 100 mM NaF opposed Mg^{++} antagonism of NaF was not determined.

TABLE XXX

ATP (mM)	Mg^{++} (mM)	NaF	% Inhibition
5	2.5	-	
	2.5	100	82
	5.0	-	
	5.0	100	76
	10.0	-	
	10.0	100	74
	20.0	-	
	20.0	100	72
	2.5	-	
	2.5	10	19
	5.0	-	
	5.0	10	36
	10.0	-	
	10.0	10	62
	20.0	-	
	20.0	10	60

Table XXX. Effects of increasing concentration of Mg^{++} on NaF inhibition of microsomal ATPase. Assays were performed as usual with added compounds as shown. P$_i$ determination was by LI method.

6) <u>NaN$_3$ Addition</u>

A final procedure which resulted in Na$^+$ stimulation
of the microsomal ATPase was addition of NaN$_3$ to the
assay medium. Some results are presented in Table XXXI.
The maximum activity ratio with .1 mM NaN$_3$ was 1.19, and
with .5 mM NaN$_3$, 1.15.

TABLE XXXI

| Na | K | .1 mM NaN$_3$ | | .5 mM NaN$_3$ | |
| | (mM) | μmoles P$_i$/mg.N/5' | | μmoles P$_i$/mg.N/5' | |
		mito.	micro.	mito.	micro.
5	.2	30.6	76.4	23.1	66.5
5	2	29.4	76.3	22.8	64.8
5	20	29.7	77.1	22.6	65.3
50	.2	30.1	82.4	22.9	71.8
50	2	30.2	83.4	23.2	70.7
50	20	28.9	82.5	23.2	71.0
100	.2	29.8	89.0	23.1	76.9
100	2	28.7	90.0	23.0	76.7
100	20	30.1	91.0	22.4	77.2
0	0	30.8	76.4	22.1	66.4

Table XXXI. Na$^+$, K$^+$ effects with NaN$_3$ on fresh microsomal
ATPase activity. Indicated concentrations of NaN$_3$, Na$^+$
and K$^+$ were added to each assay tube. [ATP]=[MgCl$_2$]=
5 mM. 50 mM histidine buffer, pH 7.2. P$_i$ determina-
tions by the LI method. Microsomal activity without
NaN$_3$ = 88.6 μmoles P$_i$/mg.N/5'. Mitochondrial activity
without NaN$_3$ = 40.7 μmoles P$_i$/mg.N/5'.

No K^+ activation could be seen, and the ouabain inhibition was still only 15% of the highest activity (i.e. not increased by Na^+ stimulation) and the glycoside had no additional inhibitory effect in the presence of submaximal concentrations of NaN_3 (Table XXXII).

However, the inhibitory effects of ouabain were decreased in the presence of maximal inhibition by NaN_3. In the presence of 1 mM NaN_3, 1 mM ouabain had no additional effect. This would suggest that ouabain and NaN_3 are acting on the same ATPase activity. However, the addition of ouabain to fresh fractions never gave Na^+ stimulation, as did NaN_3, which would be expected if ouabain and NaN_3 were acting on the same site alone, and NaN_3 allowed Na^+ stimulation.

A combination of NaN_3 and ageing gave effects comparable to ageing alone (data not shown).

Table XXXI also shows that in the presence of NaN_3, no Na^+ or K^+ effects could be seen in the mitochondrial fraction.

C. <u>Procedures Failing to Give Na^+ or $Na^+ + K^+$</u>
<u>Stimulation</u>

Since a major conclusion of this work is the absence of a K^+ stimulatory effect on the ATPase of the microsomal fraction of the rat myometrium, some of the treatments

TABLE XXXII

0 mM NaN$_3$		.1 mM NaN$_3$		.5 mM NaN$_3$		1 mM NaN$_3$	
5ATP,5Mg	5ATP,5Mg 1 ouabain	5ATP,5Mg	5ATP,5Mg 1 ouabain	5ATP,5Mg	5ATP,5Mg 1 ouabain	5ATP,5Mg	5ATP,5Mg 1 ouabain
78.8	61.4	76.4	60.6	66.4	58.4	54.0	53.8
	(21.1%)		(14.0%)		(12.1%)		(0.4%)

Table XXXII. Effects of 10^{-3} M ouabain and NaN$_3$ on fresh microsomal ATPase activity. Indicated concentrations were added to each assay tube. 50 mM histidine buffer, pH 7.2. P$_i$ determinations by LI method.

which successfully demonstrated $Na^+ + K^+$ activation in other tissues and failed to do so in this tissue will be described in detail.

A list of these procedures follows.

1) Dialysis
2) EDTA
3) Heat
4) Heparin
5) Histone (nuclear extract)
6) Isolation with various ions and metabolic inhibitors
7) Isolation in different media, and at different centrifugal forces
8) NaI
9) Sucrose gradient
10) Sonication
11) Washed microsomes

1) <u>Dialysis</u>

Microsomal fractions were isolated and homogenized as usual. The final suspension was placed in a dialysis bag, secured at both ends, and placed in a beaker with either 25 mM Tris or double distilled water. The 500 cc. beaker was then placed on a magnetic stirrer in the cold $(0-4^{O}C)$ and dialysis carried out for 12 hrs.

2) <u>EDTA</u>

(a) .1 or .5 mM EDTA was added to the sucrose iso-lating medium, and the isolation carried out as usual.

(b) .1 and .5 mM EDTA was added to the assay medium after isolation in sucrose.

3) <u>Heat</u>

(a) The usual assays were carried out at 15°C, 25°C and 50°C.

(b) The microsomal fraction was preincubated at 65°C for 10 minutes and then assayed as usual.

(c) Procedure of Somogyi (1964).

1. Microsomal fraction was isolated in .3 M sucrose plus 1 mM DOC.

2. The fraction was suspended in .3 M sucrose and heated at 65°C for 10 minutes.

3. The fraction was then incubated in .05% DOC, 5 mM $MgCl_2$ and 1 mM EDTA for 20 minutes at 65°C.

4. The suspension was centrifuged at 20,000 x g for 30 minutes.

5. The resulting pellet was resuspended in sucrose and buffer.

4) <u>Heparin</u>

.04% and .1% heparin was added to the isolating medium. It was suggested by Stahl <u>et al</u> (1963) that this compound allows cleaner separation of mitochondrial and microsomal fractions. (Effects of 1% heparin shown).

5) <u>Histones</u>

The work of Schwartz and Laseter (1964) implied that storage may allow histones which have been released during isolation to depress the Na^+, K^+ independent ATPase more than the $Na^+ + K^+$ stimulated

activity, thus increasing the activity ratio. They have shown that addition of both commercially prepared and laboratory isolated histones have a similar effect.

Commercially prepared histone was added to the assay medium, as well as to the isolation medium, in separate procedures. At concentrations of .1, .3, .5 and 1 mg/ml. there was no effect of this compound, although the higher concentrations inhibited all enzyme activity. (Effects of 1 mg/ml. histone shown)

A nuclear extract (Schwartz & Laseter,1964) obtained by heat treatment of the nuclear fraction of myometrial homogenates and presumably containing histones, was also tried in the above manner, and gave no cationic stimulation.

6) <u>Isolation with various ions and metabolic inhibitors</u>

(a) It was thought that the presence of some inorganic ions might stabilize the enzyme during isolation. Thus isolation at the usual speeds were performed with the addition of 5 mM $MgCl_2$, 100 mM NaCl and 20 mM KCl to the isolation medium. Subsequent assay of the microsomal fraction gave no cationic effects.

(b) DNP (1 mM) and (c) NaF (10 mM) were also tried in the same manner without success.

7) <u>Isolation in different media and at different centrifugal forces</u>

(a) The following media were used for isolation in place of .25 M or .3 M sucrose. In no case was there any

effect on the cationic stimulation of the microsomal fraction, except in the latter treatment.

1. 30 mM histidine
    5 mM EDTA

2. .3 M sucrose
    30 mM histidine
    5 mM EDTA

3. .3 M sucrose
    30 mM histidine
    5 mM EDTA
    .1-.25% DOC

This last medium gave slight Na^+ stimulation, due to the presence of DOC.

(b) The mitochondrial fraction was spun at 36,000 x g (20,000 rpm) instead of the usual 18,000 x g (13,500 rpm). It was considered that the microsomal fraction could contain mitochondria, and the ATPase associated with these organelles could obscure Na^+ and K^+ stimulation. Centrifugation of the mitochondrial fraction at higher speeds would presumably give less mitochondria in the subsequent microsomal fraction.

Table XXXIII shows the results of such treatment, and depicts the oxygen uptake and ATPase activity after mitochondria. have been isolated at different speeds. The microsomal fraction remaining after the high speed mitochondrial isolation still did not show Na^+ and K^+ effects. Furthermore, less oxygen uptake was demonstrated in this fraction, suggesting less mitochondrial contamination.

TABLE XXXIII

Microsomal Fraction	mμmoles O_2/mg.N/min.	μmoles P_i/mg.N/5'		
	O_2 uptake	5 Mg	5Mg,100Na (mM)	5Mg 100Na,20K
Mitochondria (Heavy Microsomes) After 18,000 x g	100	86.7	87.5	86.8
Mitochondria (Light Microsomes) After 36,000 x g	60	71.4	70.7	70.8

Table XXXIII. Relation between O_2 uptake, centrifugal force of mitochondrial isolation, and enzyme activity of fresh microsomal fractions. The "heavy" and "light" microsomes are the microsomes isolated after two different mitochondrial isolation procedures. Enzyme activity was measured as μmoles P_i/mg.N/5' in 50 mM histidine buffer, pH 7.2, using the LI method of P_i determination. (N=4). The nitrogen content of the light microsomes was found by the Kjeldahl method to be .96 mg.N/gm. wet weight tissue as opposed to 1.2 mg.N/gm. wet weight tissue. The O_2 uptake was measured in the medium described in Table II.

8) NaI

Two different procedures were tried using NaI, which for other tissues gave a highly specific Na^+ - K^+ activated ATPase.

(a) Nakao et al (1963).

1. To 4 ml. of microsomal suspension, 4 ml. of a NaI solution was added. This solution contained 6.6 M NaI,

50 mM cysteine, 5 mM $MgCl_2$, 3 mM ATP and 5 mM EDTA. The solution was added in the cold.

2. After 30 minutes the suspension was diluted to 10 cc. with double distilled water, centrifuged at 115,000 x g, and the pellet resuspended in histidine buffer.

(b) Matsui and Schwartz (1966) tried a combination of DOC and NaI. This procedure was slightly altered for use on rat myometrium, and the microsomal fraction was isolated in .1% DOC, and then treated as above with NaI. Preliminary studies only were carried out using this procedure.

9) Sucrose Gradient Centrifugation

Suspensions of microsomes were placed on a layered sucrose density gradient, ranging from 1.0 M to 1.6 M. This treatment gave 2 separate bands using the SW39-L "swinging bucket" rotor, one in the 1.0 M layer, and one in the 1.4 M layer. The heavier material had very little ATPase activity, but the lighter layer had much ATPase activity, none of which appeared to be Na^+ or $Na^+ + K^+$ sensitive. A few such studies of normally isolated, stored microsomes were carried out, without giving any additional cationic effects.

10) Sonication

A freshly isolated microsomal fraction was treated with a Raytheon Sonic Oscillator for 30 secs. and 60 secs. The resulting materials were recentrifuged at 127,000 x g (37,500 rpm) for 1 hr., and the resulting pellet resuspended for assay. The results showed only less activity of the treated

microsomes both with and without addition of Na^+ and K^+.

11) Washed Microsomes

The microsomal fraction was washed 2 times to try to decrease the amount of Na^+ and K^+ bound to the fraction, which may be responsible for the lack of Na^+ and K^+ stimulation (Jarnefelt, 1964). The results are shown in Table XXXIV, and demonstrate that 80% of the K^+ and 60% of the Na^+ could be washed off, but neither of these ions alone or together gave stimulation in fractions subsequently tested.

TABLE XXXIV

	Nuclear			Mitochondrial			Microsomal		
Wash	Ca	Na	K	Ca	Na	K	Ca	Na	K
Control	.15	1.7	.42	.26	1.3	.15	.18	2.11	.53
1	.14	1.4	.17	.10	.9	.06	.14	1.18	.18
2	–	–	–	–	–	–	.13	.83	.10
% of original	94	82	40	30	69	40	72	39	19

Table XXXIV. Effects of "washing" on electrolyte content of subcellular fractions of rat myometrium. After each fraction was isolated in the usual manner, it was resuspended and recentrifuged twice, in the case of microsomes. The values are in μmoles of electrolyte per fraction. Each washing caused loss of electrolyte, and some loss of solid material. The percentages indicated are the amount of electrolyte remaining in the pellet after the wash procedure (N=3).

A brief summary of the experimental data found from all these procedures is seen in Table XXXV.

VII. <u>Effects of Preincubation with NEM, NaF, Urea and ATP, and Fresh Microsomal ATPase</u>

Skou and Hilberg (1965) have demonstrated that the preincubation of brain and kidney microsomes with specific compounds can increase the activity ratio. However, if these fractions were preincubated with these specific compounds plus ATP, the activity ratio was increased even more than without the nucleotide.

Similar preincubations were tried on this tissue. The fractions were isolated and homogenized as usual. 1 cc. aliquots of the homogenate were then placed in separate test tubes, and to these portions were added the various compounds to be preincubated with the fraction. After the preincubation (usually 15'), .2 cc. portions of the preincubation mixture were withdrawn, and placed in the various assay media.

The following diagram illustrates the procedure, with x equal to NEM, NaF or urea:

TABLE XXXV

Procedure	(mM)	5 Mg	5 Mg, 100 Na	5 Mg, 20 K	5 Mg, 100 Na, 20 K
1) Dialysis*	C	2.05	2.08	–	2.06
	T	1.97	1.93	–	1.91
2) EDTA	C	94.7	94.9	95.0	95.2
	T	a 93.6, b 94.9	a 93.8, b 94.2	a 93.6, b 94.1	a 93.0, b 94.0
3) Heat	C	88.4	85.2	–	–
	T	a 73.7, b 63.1, c 0	a 74.2, b 55.0, c 0	a 74.6, b 59.1, c 0	a 74.0, b 60.0, c 0
4) Heparin	C	82.4	82.5	82.0	82.3
	T	80.6	80.7	80.6	81.1
5) Histone	C	83.6	84.8	84.2	84.0
	T	64.8	65.3	65.0	65.1
Nuclear Extract	C	89.8	88.9	88.1	87.3
	T	70.2	71.7	70.6	71.4
6) Isolation	C	–	–	–	–
	T	a 78.7, b 70.8, c 67.1	a 78.4, b 70.1, c 67.0	–	a 78.0, b –, c 66.8
7) Isolation (a)	C	96.3	96.2	–	96.2
	T	1 94.7, 2 95.9, 3 86.4	1 94.1, 2 94.0, 3 96.3	–	1 94.0, 2 93.1, 3 94.9
Isolation (b)	C	86.7	87.5	–	88.1
	T	71.4	70.7	–	70.8
8) NaI*	C	–	–	–	–
	T	a .74, b 1.08	a .71, b 1.07	–	a .73, b 1.09
9) Sucrose Gradient*	C	2.70	2.58	–	–
	T	.83	.80	–	2.71, .81
10) Sonication*	C	1.17	1.15	1.20	1.17
	T	1.02	1.03	1.09	1.00
11) Washed Microsomes*	C	1.81	1.86	1.80	1.77
	T	1.21	1.20	1.20	1.18

Table XXXV. Summary of procedures not giving Na$^+$ stimulation. In each assay [ATP] was always 5 mM. Buffer was always 50 mM histidine, pH 7.2. Pi determinations were done with both LI and FS methods. In procedures marked with asterisks (*) Pi determinations were measured in μmoles Pi liberated/test (3 cc. assay medium)/5 min. C=control, T=treated. See text for details.

(1)

                    total microsomal
                       suspension

              1 cc. 1 cc. 1 cc. 1cc.

(2)

              X   ATP   X+ATP   Control

       .2 cc. .2 cc. .2 cc. .2 cc. .2 cc.   .2 cc.
                                         .2 cc. .2 cc.

(3) 5ATP   5ATP   5ATP   5ATP   5ATP   5ATP   5ATP   5ATP
     5Mg   5Mg   5Mg   5Mg   5Mg   5Mg   5Mg   5Mg
             100Na   100Na   100Na   100Na
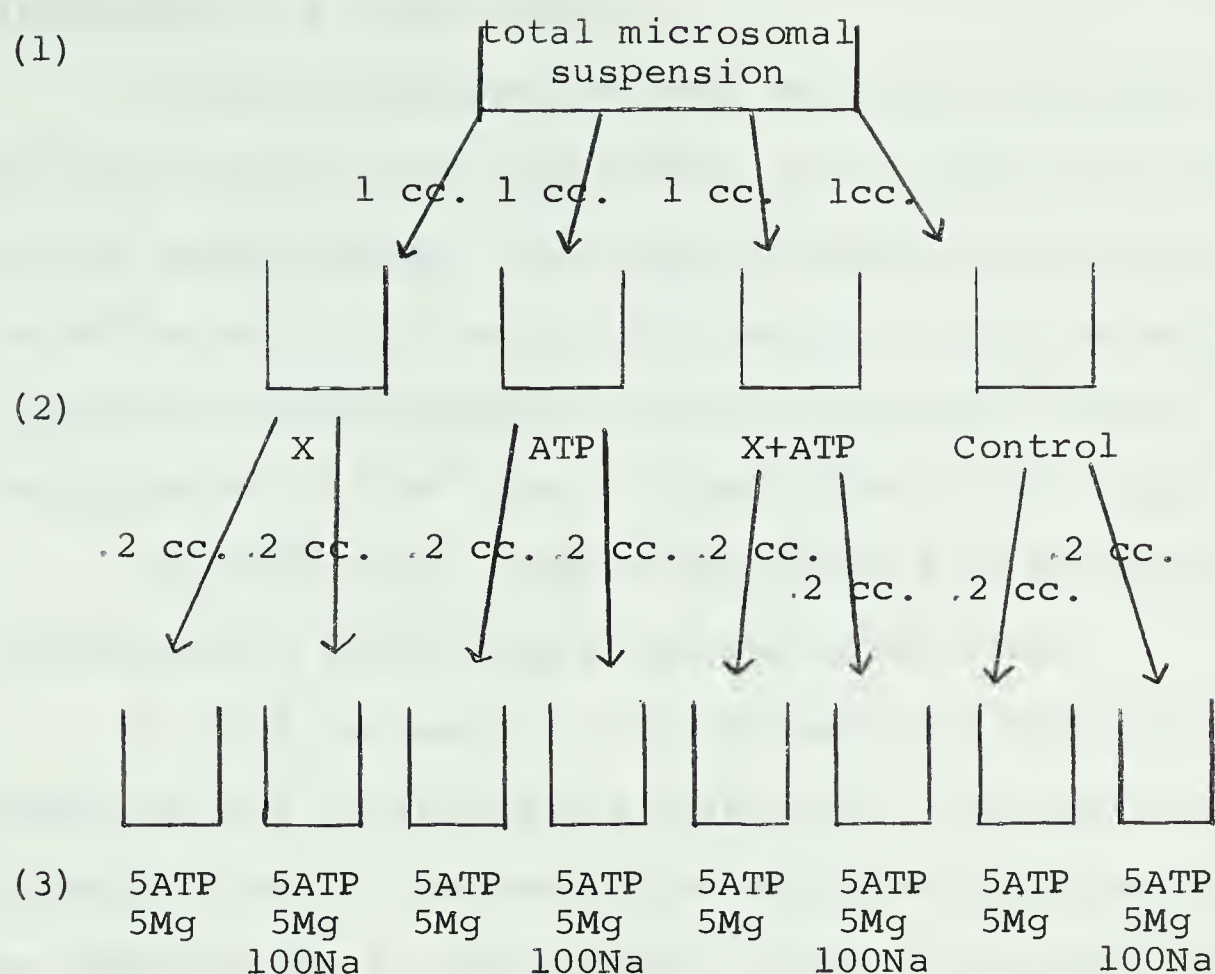
Step (1) is isolation of the regular microsomal suspension.

Step (2) is the preincubation procedure, lasting 15 minutes unless otherwise indicated.

Step (3) is the usual assay procedure, with the indicated assay media.

Table XXXVI is a summary of these results.

The data demonstrate that preincubation with NEM, NaF, or urea either with or without ATP did not allow subsequent Na^+ stimulation. Preincubation with ATP also caused no Na^+ stimulation. However, ATP prevented the inhibitory effect of each of these 3 compounds on the Mg^{++} stimulated ATPase activity. The possible mechanisms involved will be

discussed in a later section.

It was interesting to note that preincubation with
NaF did not have the same effect as the addition of NaF
to the assay medium. The former treatment did not result
in Na^+ stimulation, as did the latter. Other experiments
showed that preincubation with NaF prevented the Na^+ stimu-
latory effect of Na^+ when NaF was added to the assay medium.

The difference between the effects of NaF exposure on
preincubation and during assay may arise from:

1) The necessity of the presence of NaF during the
assay.(If NaF inhibition is reversible, NaF might cause an
effect during the preincubation which might subsequently
be reversed due to the absence of NaF in the assay medium).

2) The necessity of the presence of Mg^{++} or Na^+ or
both together with the NaF to produce any activation effect.

3) The necessity of ATP splitting in the presence of
NaF, or the simultaneous presence of ADP or P_i with NaF for
appearance of Na^+ activation.

4) The damaging effect of the preincubation procedure
itself.

5) The damaging effect of the NaF during preincu-
bation.

Experiments were designed to test these possibilities.
They were similar to the preincubation studies previously
outlined, but for 2 differences; 1) only 2 preincubations

TABLE XXXVI

Preincubation Assay	5 mM ATP		"X"		"X" + ATP		Control	
	5 Mg	5Mg,100Na	5 Mg	5Mg,100Na	5 Mg	5Mg,100Na	5 Mg	5Mg,100Na
X = 1 mM NEM	74.0	71.7	41.3	42.0	60.4	60.0	71.0	70.8
X = 10 mM NaF	105.1	95.4	51.0	47.3	75.1	75.7	109.4	110.7
X = 5.0 M urea	70.5	70.7	24.1	24.7	69.7	69.5	67.4	67.7

Table XXXVI. Effects of preincubation and ATP protection on fresh microsomal ATPase. The top column shows the preincubation compounds; ATP, "X", ATP + "X", and control (incubation in 50 mM histidine buffer, pH 7.2 only). The column on the left shows the three different compounds that were "X". The assays were done in histidine buffer, pH 7.2, with [ATP] = 5 mM, and the LI method used for P_i determinations. For further details, see the text. NEM, N=3, NaF, N=6, Urea, N=2.

were involved, and 2) the effect of Na^+ addition was tested
in the presence and absence of 10 mM NaF in the assay medium.
The experimental design and results are summarized in Table
XXXVII.

The following chart shows 4 of these 5 possibilities
have been eliminated.

Possibility	Eliminated by Experiment No.
1	1,6
2	2
3	1,4,5
4	3
5	-

The first possibility was eliminated by experiments
1 and 6. If this possibility were true, preincubation in
NaF, and subsequent assay in the presence of NaF should
give stimulation. This did not happen in experiments 1
or 6.

The second possibility was eliminated by experiment
2, since preincubation in the presence of $MgCl_2$, NaCl and
P_i, with and without NaF, gave no Na^+ stimulation when
the fraction was subsequently assayed with and without
NaF.

TABLE XXXVII

Preincubation	No NaF in assay		10mM NaF in assay	
	5Mg	5Mg,100Na	5Mg	5Mg,100Na
10 mM NaF	97	96	67	69
10 mM NaF + 5 mM ATP (1)	133	131	96	96
5mM MgCl$_2$+100mM NaCl+5mM P$_i$+10mM NaF	99	80	57	58
5mM MgCl$_2$+100mM NaCl+5mM P$_i$ (2)	116	118	100	97
Control	91	93	61	77
10 mM NaF (3)	71	68	34	36
5 mM P$_i$	81	79	57	56
10 mM NaF + 5 mM P$_i$ (4)	53	46	43	42
5 mM ADP + 5 mM P$_i$	89	87	49	51
5 mM ADP + 5 mM P$_i$ + 10 mM NaF (5)	74	73	72	70
10 mM NaF. 15 min.	82	83	71	69
10 mM NaF. 5 min. (6)	91	91	70	70

Table XXXVII. Effects of preincubation and treatment with NaF on fresh microsomal ATPase. This table indicates 6 separate experiments. In each case, the preincubations were different as indicated. The assays were always the same; 5 mM Mg^{++}, 5 mM Mg^{++} + 100 mM Na$^+$, in 50 mM histidine, pH 7.2. [ATP] = 5 mM. P$_i$ determinations were done by the LI method.

The third possibility was eliminated by experiments 1, 4 and 5, since preincubation with ADP, P_i and ATP, with and without NaF allowed no Na^+ stimulation.

The fourth possibility was eliminated by experiment 3, since control incubation (i.e. incubation of the microsomal suspension in histidine buffer only) still allowed Na^+ stimulation when the fraction was subsequently assayed in NaF.
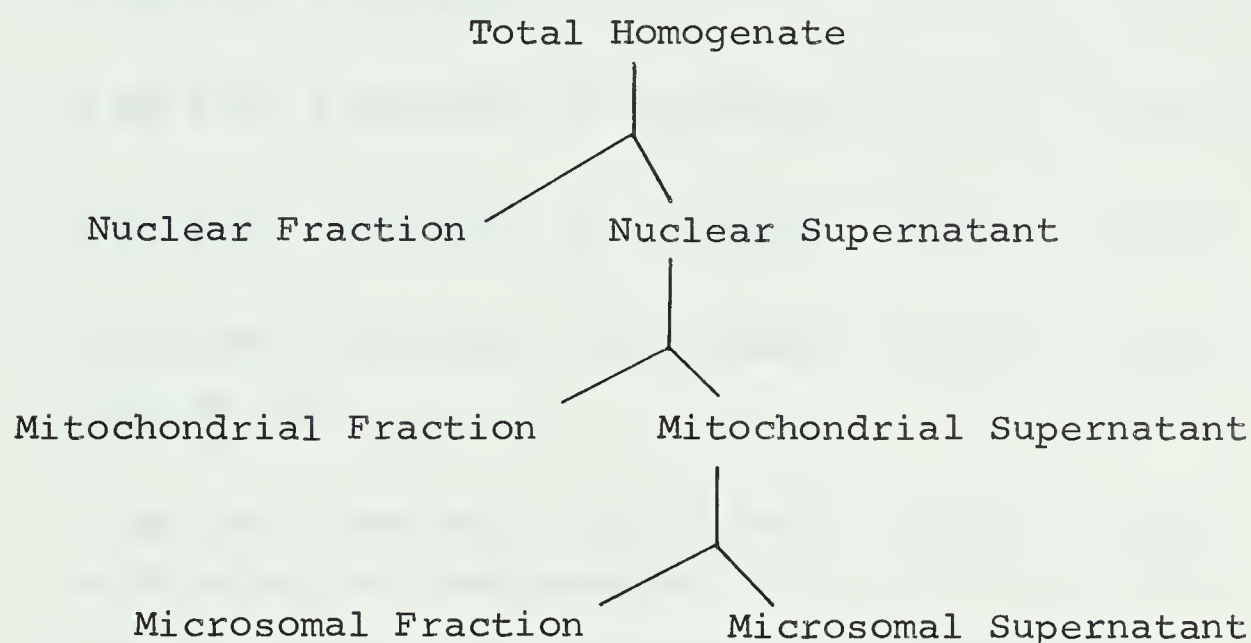
When the preincubation time in the presence of NaF was lowered from 15 minutes to 5 minutes, there was still no Na^+ activation. Thus it appears that even a brief preincubation of the enzyme with F^- does something to the system so that NaF no longer gives Na^+ stimulation (possibility 5). The mechanism of this effect, and the critical time required for the mechanism are unknown.

VIII. <u>Fate of Na^+ Activated Ouabain Inhibited ATPase of the Fresh Homogenate</u>.

In the first section of this thesis, it was demonstrated that the fresh total homogenate of rat myometrial tissue contained a Na^+ activated ouabain inhibited ATPase activity. However, this activity was not demonstrable in fresh microsomal material and certain treatments were required to allow Na^+ to exert its activating effect. The reasons for this alteration have not been completely elucidated, but some pertinent information has been obtained.

As Table VI shows, the nuclear supernatant contains enzyme activity similar to that of the fresh total homogenate. Thus as previously shown, the Na^+ activated ATPase activity is not lost in the nuclear fraction.

A schematic representation of microsomal isolation follows.

Total Homogenate

Nuclear Fraction   Nuclear Supernatant

Mitochondrial Fraction   Mitochondrial Supernatant

Microsomal Fraction   Microsomal Supernatant

Separation of the nuclear supernatant into the mitochondria and mitochondrial supernatant resulted in a loss of the Na^+ activated ATPase activity in these fresh fractions. It has previously been shown that no treatments of the mitochondria could show a Na^+ activating effect.

However, ageing treatment of the mitochondrial supernatant gave Na^+ activation as in the microsomal fraction (Table XXXVII) and no K^+ effect.

Thus the activity of the microsomal fraction is, as expected, present in the mitochondrial supernatant. Now

TABLE XXXVIII

|	Fresh	Aged
5 mM ATP, 5 mM MgCl	2.74	1.98
5 mM ATP, 5 mM MgCl, 1 mM ouabain	2.53	1.81
5 mM ATP, 5 mM MgCl, 100 mM NaCl	2.73	2.61
5 mM ATP, 5 mM MgCl, 100 mM NaCl + 20 mM KCl	2.71	2.59
5 mM ATP, 5 mM MgCl, 100 mM NaCl + 20 mM KCl + 1 mM ouabain	2.22	1.80

Table XXXVIII. Effects of ageing on mito-
chondrial supernatant ATPase activity.
.2 cc. of the suspension was used in each
assay. Aliquots were taken at 5 minutes
for P_i determination by LI method. Acti-
vity is expressed as μmoles P_i/3 cc. assay
medium/5'. Histidine buffer (N=5).

the question arises "What has happened to the activity of the nuclear supernatant?" There are 3 possibilities; 1) something is lost to the mitochondrial fraction, 2) there is an alteration in the enzyme during the isolation, or 3) something is gained from the mitochondria causing an alteration in the enzyme.

The first possibility was tested by adding the mitochondrial fraction back to the mitochondrial supernatant and microsomal fraction. Careful controls were run because of the ATPase activity present in the mitochondria. No Na^+ activation was noted in either the fresh microsomal fraction or mitochondrial supernatant. Some experiments were tried in which the mitochondrial fraction was boiled, or to which NaN_3 was added to inhibit the mitochondrial ATPase. Again, no Na^+ activation was demonstrable. Thus tentatively it may be said that material is not lost to the mitochondria to cause a disappearance of Na^+ activated ouabain inhibited activity.

Possibilities 2 and 3 are more difficult to test. Ideally, if something is gained from mitochondria during isolation to alter the enzyme activity, mitochondria at some stage of isolation should no longer contain the unknown compound. Thus the addition of mitochondria from various times of isolation to treated microsomes could show different effects. Addition of completely isolated

FIGURE 29
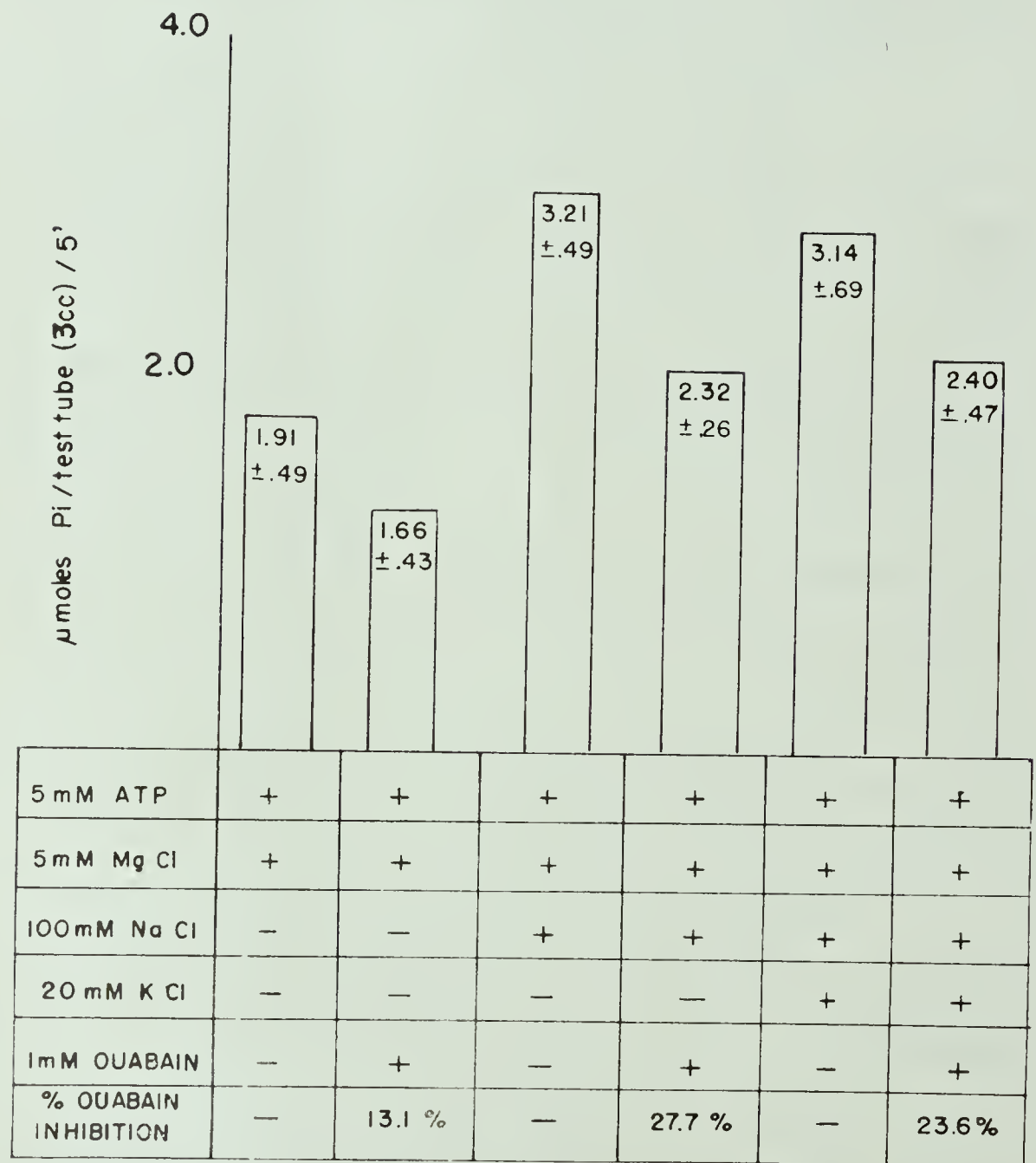
mitochondria to aged microsomes and aged mitochondrial supernatant did not alter the Na^+ activation to any greater extent than could be expected from the ATPase activity present in the mitochondria. This was merely an additive effect.

VI. Studies with the Isolated Plasma Membrane

A useful method of corroborating the lack of K^+ stimulation on the ATPase of myometrial microsomes would be to demonstrate a similar effect on the plasma membrane of myometrial cells, isolated by a completely different procedure. Tests for Na^+ and K^+ effects were carried out on such a fraction. The results can only be expressed in μmoles P_i/ test tube since nitrogen content or some other quantitative unit was not measured for these preparations. Three separate membrane fractions were studied at different times, and the combined results are presented in Figure 29.

The data show that in the plasma membrane preparation, Na^+ stimulated the ATPase activity giving an activity ratio of 1.6. K^+ had no stimulatory effect, and ouabain inhibited the activity more when Na^+ stimulation occurred. Although no gross mitochondrial contamination of this preparation was visible under the electron microscope, the fraction still exhibited considerable oxygen uptake. Consequently, it is difficult to draw conclusions about the contamination

Figure 29. Na^+ stimulation, ouabain inhibition and lack of K^+ effect on ATPase of membrane preparations. The preparation was suspended in histidine buffer, pH 7.2. .4 cc. of this suspension was used in each assay. P_i determination by FS method. Activity expressed as μmoles P_i/test tube/10', ± standard deviation.
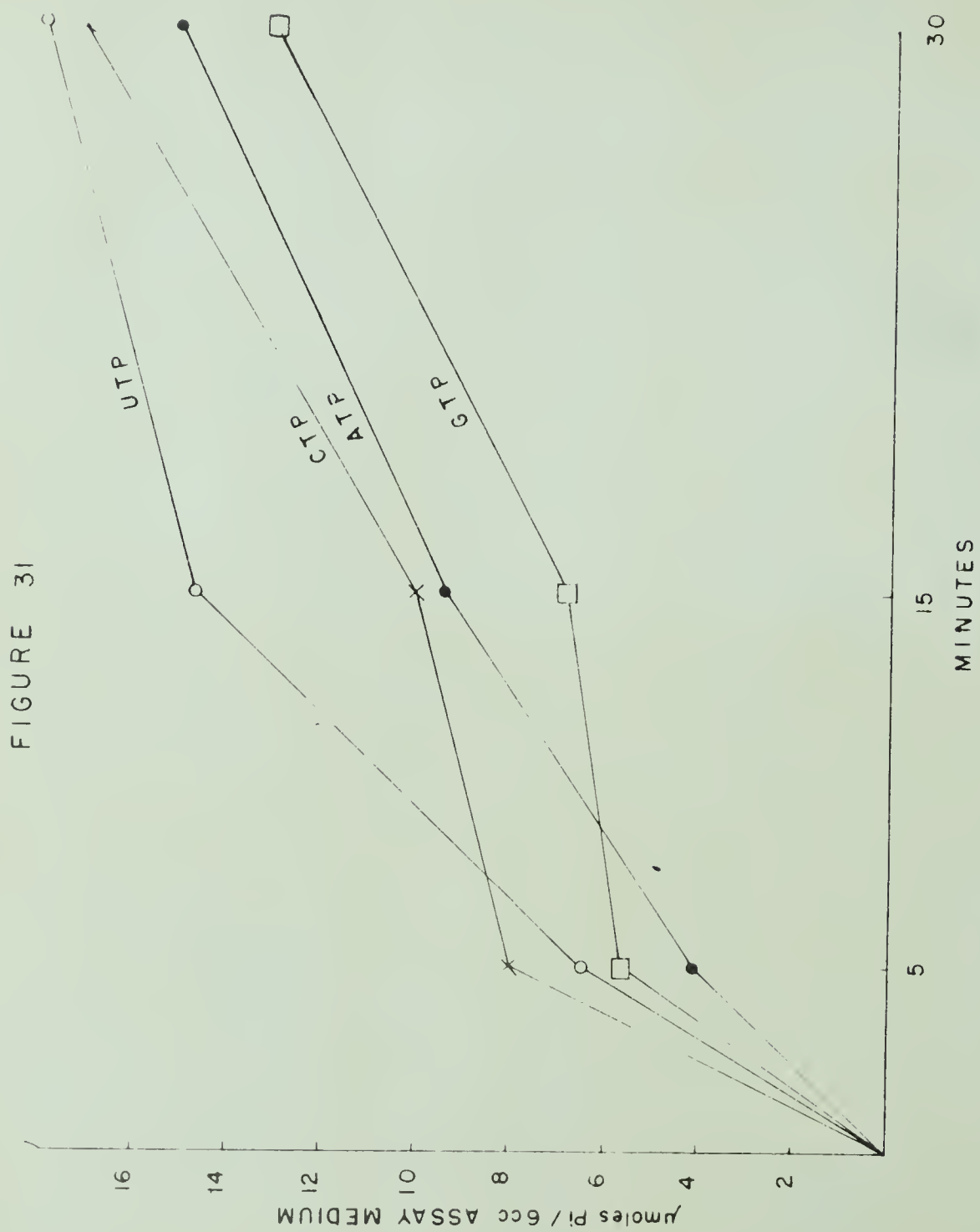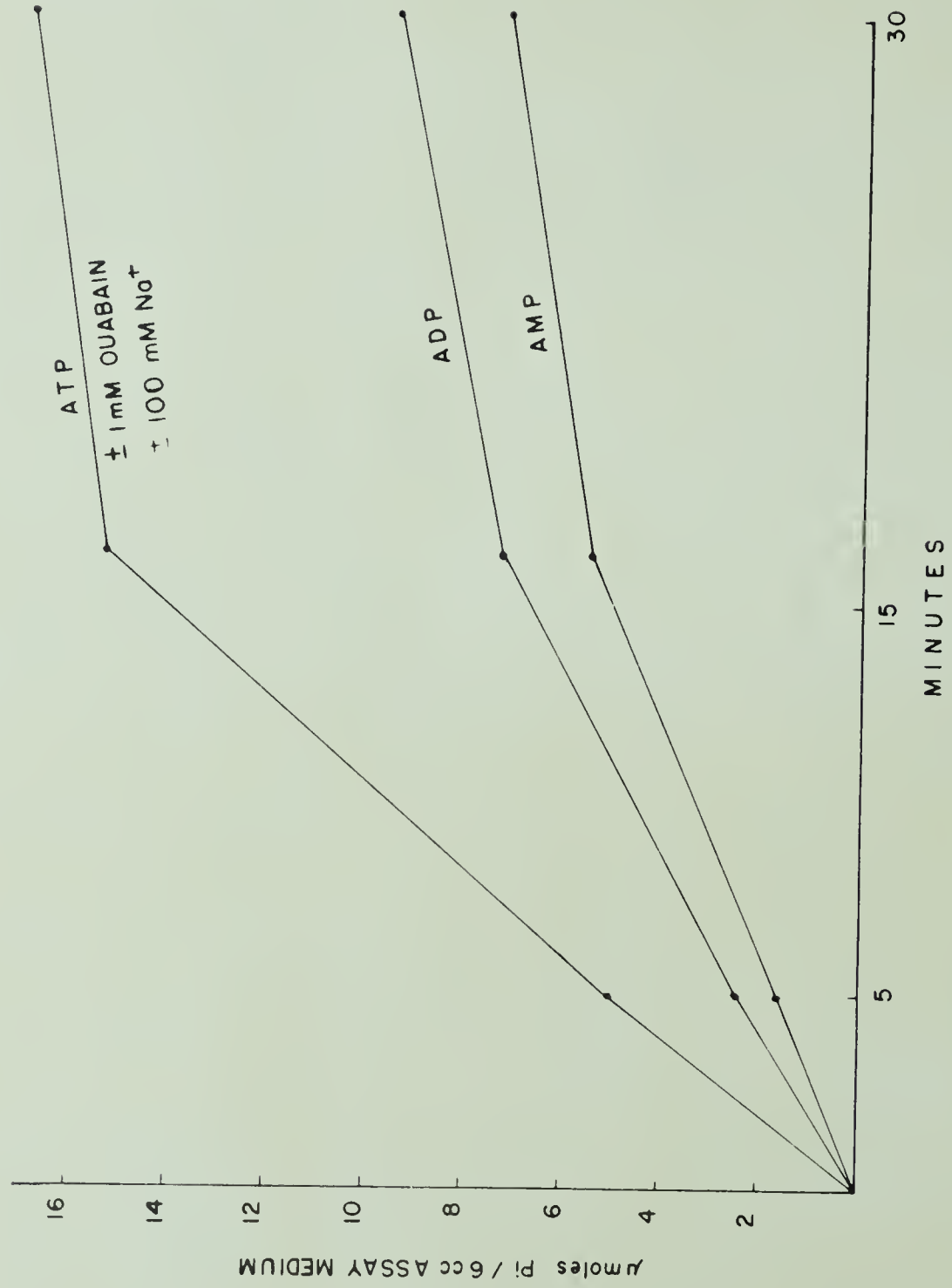
FIGURE 31

FIGURE 30

of this preparation.

X. Studies with Intact Tissue

Since the rat uterus contains a very high ATPase
activity, and since various compounds and ions which in-
hibit the ATPase activity can also cause contraction
(ouabain, NaF, $CuCl_2$), the enzyme activity of the intact
tissue was determined. This was done by immersing the
tissue in phosphate-free Krebs, adding ATP and various
other nucleotides and measuring P_i in the medium.

Uteri were excised as usual, and placed in cold
phosphate-free Krebs. This medium was then incubated
at $37^{\circ}C$ for 5 minutes, and then 30 μmoles of the nucleo-
tide in question was added. At various time intervals
aliquots were removed, placed in cold 15% TCA, centrifuged
and the P_i determined.

The results in Figures 30 and 31 show that ATP and
other triphosphonucleotides are rapidly hydrolyzed as in
tissue homogenates and the microsomal fraction. These
similarities extend also to a faster hydrolysis of ATP

Figure 30. Hydrolysis of adenine nucleotides by intact
rat uterus. Nucleotide concentrations were always 5
mM in 6 cc. phosphate free Krebs. Intact uteri were
placed in the media, and 1 cc. aliquots were withdrawn
as usual. P_i determinations were done with FS and LI
methods. Test tubes were stoppered, so in spite of
lack of oxygenation, there was no large pH change (N=3).

Figure 31. Hydrolysis of trinucleotides by intact rat
uterus. Same as Figure 30 (N=3).

than of ADP, although AMP was not hydrolyzed at all in the microsomal fraction. Neither 100 mM Na^+ nor 1 mM ouabain had inhibitory effects on the ATP hydrolysis in the intact tissue (when Na^+ was to be tested, Na^+ was replaced in the Krebs by sucrose).

Adenosine gave no appreciable P_i, nor did tissue incubated without NTP, so the hydrolytic effect was not an artefact. The tissue contains a non specific nucleoside-triphosphatase, and can also hydrolyze ADP and AMP as well as ATP. None of the nucleotides were hydrolyzed to any extent in Krebs in the absence of tissue, nor was the fluid in which tissues were bathed for up to 1 hour capable of causing nucleotide hydrolysis, indicating that leakage of enzymes from damaged cells could not have accounted for these results.

DISCUSSION AND CONCLUSIONS

I. Use of Differential Ultracentrifugation

The procedure of differential ultracentrifugation has been used often for the isolation and study of various subcellular components (Allfrey, 1960; Schneider and Kuff, 1964). The technique was used in this thesis to study the fractions of rat myometrial tissue, and its limitations should be briefly discussed.

1. The separation is based upon the buoyant density of the subcellular particles, and the particles from various cellular locations may be present in overlapping densities. As a result, the various isolated fractions are almost certainly impure.

2. The extent and nature of the homogenization procedure will affect the size and range of densities of particles from each cellular locale. This would also lead to impure and variable fractions.

3. Particle aggregation, adsorption of unrelated cellular material, particle lysis and other post-homogenization procedures will probably alter particle densities as well as alter the enzymic constituents.

4. The activity or function of a cell structure may be damaged or altered by homogenization and isolation.

As a result, the presence or absence of a given activity in a fraction cannot be taken as proof that the

activity existed or was absent in the comparable cellular structure in the intact cell, and other evidence is needed to supplement these studies to demonstrate the extent of contamination, if possible. These procedures; variation of isolation and homogenization procedures, variation of forces used for separation, studies of enzymatic activities in different fractions, etc. have been described in the results sections and will be discussed further in this section. Possible alteration of an ATPase activity or the function of an enzyme during isolation has been evaluated by comparing their activities in whole myometrial homogenates and isolated preparations in the expectation that results common to a variety of procedures are probably not procedural artefacts.

II. Characterization of Mitochondrial and Microsomal Fractions

In this work, a number of criteria have been used to attempt to characterize the three subcellular fractions. It should be noted that while sucrose is the best isolation medium for conserving both the microsomes and mitochondria, this medium disrupts intact nuclei (Schneider and Kuff, 1964) possibly causing release of nuclear

material, and subsequent deposition in other isolated frac-
tions.

Uterine smooth muscle has little endoplasmic reticu-
lum (Mark, 1956; Schoenberg, 1958). It has been shown
that skeletal muscle microsomes consist largely of endo-
plasmic reticulum (see review, Siekevitz, 1963).

The electron micrographs of rat myometrial tissue of
Wakid (1960) show granulated microsomal vesicles, and the
question arises, "What has been photographed?" It is
tempting to suppose that since this tissue contains only
sparse endoplasmic reticulum, and since the microsomal
fraction presumably contains most of the cell's membranous
material that this fraction contains chiefly plasma mem-
brane. This hypothesis could have been grossly tested by
weighing microsomal and membrane preparations in relation
to total tissue wet weight.

Wakid and Needham (1960) found different nitrogen
distribution in the various subcellular fractions of uteri
from pregnant and ovariectomized treated rats than I
found in fractions from intact treated animals. Unfortun-
ately, no comparison of absolute values is possible, since
these workers did not state the weight of the myometrium
used for nitrogen analysis.

They found the microsomal fraction always contained
more nitrogen than the mitochondrial fraction. In contrast

(Table IV)   I found that the mitochondrial fraction con-
tains more nitrogen (3.21 mg. N/gm. wet weight of uterus)
than the microsomal fraction (1.2 mg. N/gm. wet weight of
uterus).

These workers further stated that although uterine
mitochondria could oxidize substrates of the Krebs cycle
well, no oxidative phosphorylation could be demonstrated
using ADP as acceptor. I found comparable oxygen uptake
values using succinate (200 mμmoles O_2/mg. N/minute = 270
μl. O_2/mg. N/hour.. Their results were 289 μl. O_2/mg. N/
hour with succinate), and some indication of respira-
tory control with ADP, suggesting the presence of oxida-
tive phosphorylation.

I have also shown that the microsomal fraction has
considerable oxidative activity. This could indicate
either a large contamination of the microsomal fraction
with mitochondria or the presence of oxidative activity
in microsomes. Siekevitz (1963) and others have shown
that microsomal fractions of tissues that contain exten-
sive endoplasmic reticulum have large oxidative activities,
although the success of succinate as electron acceptor has not
been evaluated. Other data also indicate that this
electron transport system is distinct from that of
mitochondria.

In the rat uterus, exclusion of "heavy microsomal"

material by centrifugation at increased speeds, lowered
the oxygen uptake of subsequently isolated microsomes
(Table XXXIII). This observation, coupled with the fact
that the endoplasmic reticulum is sparse in myometrial
tissue, suggests that a large part of the oxidative
activities of the heavy microsomal fraction is due to
mitochondrial contamination. The use of oxidative
activity in this manner illustrates how the study of a
specific enzyme system may lead to conclusions about
the relative purity of subcellular fractions.

Another enzyme marker used in this study was glu-
cose-6-phosphatase, which is presumably located in the
microsomal fraction (Roodyn, 1965). Assays of this
enzyme (page 97 of results) showed negligible quantities
present in any fraction but the microsomal, suggesting
that microsomes do not contaminate the mitochondrial
fraction.

Electron micrographs are also of considerable value
in determining fraction purity. Such pictures of rat
myometrial subfractions show no gross mitochondrial con-
tamination of the heavy microsomal fraction, but mito-
chondrial fragments could sediment along with the mem-
branes of endoplasmic reticulum during heavy microsomal
isolation. If the "light microsomes" were relatively
less contaminated with mitochondria, then ATPase activity

in this fraction should be less contaminated by mitochond-
rial ATPase activity. This was true, since total ATPase
activity of the light microsomes was reduced.

III. Difficulties of Contamination

Contamination of subcellular fractions can lead to
difficulty in interpretation of results. Samaha and
Gergely (1966) showed that with NaN_3 and ouabain together,
all the ATPase activity of human skeletal muscle microsomes
was inhibited, but that only ouabain could inhibit the Na^+
+ K^+ activated portion. Some Na^+ and K^+ activation was
present even without the addition of NaN_3. The presence
of this compound inhibited the mitochondrial ATPase and
allowed an increase in the relative Na^+ + K^+ activation
of microsomal ATPase. Their suggestion was that the
basic Mg ATPase activity of the microsomes was all due
to mitochondrial contamination.

This interpretation is complicated by the data of
Auditore and Wade (1964) who found a Na^+ + K^+ - Mg
ATPase activity in rabbit kidney mitochondria. It is
possible that the mitochondria were contaminated by
heavy microsomes since only cytochrome oxidase measure-
ments were carried out, indicating little contamination
of microsomes by mitochondria but not eliminating the
reverse contamination. The Na^+ + K^+ activated ATPase
activity was inhibited by ouabain. NaN_3 was required

to show $Na^+ + K^+$ activation, since without it, Na^+ and K^+ inhibited the mitochondrial ATPase.

Sugawara and Utida, (1963) also found Na^+ and K^+ activated ATPase in brain mitochondria, but used only succinic dehydrogenase as a test of fraction purity. The activity of this preparation was not characteristic of concurrently studied microsomal ATPase in that K^+ could activate the enzyme alone. Ouabain was not tested, although Schwartz et al (1962) showed inhibition of brain microsomal ATPase by the glycoside.

IV. Mg^{++} Requiring ATPase

The presence of a Mg^{++} sensitive ATPase is well established in both liver microsomes (Ernster and Jones, 1962) and liver mitochondria (Ulrich, 1964, 1965, 1966). Rat myometrial fractions contain comparable activities (Wakid, 1960; Wakid and Needham, 1960).

The present work has demonstrated that both these fractions possess an ATPase activity which requires Mg^{++} for maximum activation, the optimal Mg^{++}/ATPase ratio being 1/1. As soon as this ratio is altered either by an increase or decrease of Mg^{++} or ATP, the enzyme activity decreases.

The most unusual feature of the microsomal Mg ATPase activity of this tissue is the very fast initial

rate of hydrolysis, averaging over 200 μmoles P_i/mg. protein/hour.

Below is a table of ATPase activities of some other tissues studied, using similar isolation (differential ultracentrifugation) procedures.

| Source of Microsomes | (range) μmoles P_i/mg.P/hr. | | Reference |
	Mg	Mg+Na+K	
Brain			
guinea pig	70-88	316-344	Schwartz (1964a)
Cardiac Muscle			
guinea pig	39-75	52-80	Schwartz (1964a)
rat	2	2	Schwartz (1962)
Skeletal Muscle			
human	3-9	12-18	Samaha & Gergely (1966)
frog	95-99	141-145	Duggan (1964)
rabbit	300-420	650-780	Fratantoni & Askari (1965)
Kidney			
guinea pig		50-300	Post et al (1965)
Liver (rat)	4-8	23-30	Charnock & Post (1963)
	23-30	98-106	Schwartz (1964)
Rat Myometrium	175-200	175-200	This thesis

Only one value of Mg ATPase activity in this chart is larger than that obtained for rat myometrium.

Another unusual feature of myometrial tissue lies not only in its high activity but also in the fact that hydrolysis becomes non-linear with time at the end of only 5 minutes. This may be due in part to the enzyme system's susceptibility to ADP inhibition.

A. K_M Value

The K_M value for the basic Mg ATPase of .57 mM for this tissue is similar to K_M's found for other tissue (cardiac muscle, .24 mM. Matsui and Schwartz, in press). The significance of K_M values for particulate enzymes is uncertain.

B. pH Curve

The pH activity curve of the microsomal ATPase was very broad, and the pH range over which the ATPase activity was highest was 7-8 (values over 8 were not studied). A similar curve was found by Samaha and Gergely (1966) for the Mg ATPase of skeletal muscle microsomes. However, addition of Na^+ and K^+ to their system altered the pH curve giving a sharp optimal pH of about 7.5 as well as stimulating the activity.

Effects of pH on stored microsomal fractions were performed with the Mg^{++}/ATP ratio of 1/1. The pH-activity

curve without Na^+ present was similar to that of fresh microsomes (Figure 28). The addition of Na^+ to stored microsomes, showed a much sharper pH curve. The difference between these two curves shows that the Na^+ activated ATPase of stored microsomes has a peak between pH 7.2-7.4 (Figure 28).

C. Mg^{++}, ATP and ADP Inhibition

Throughout this paper it has been assumed that Mg ATP is the normal substrate for the ATPase. Figures 10 and 13 show clearly that excess Mg^{++}, ATP or Mg ATP can inhibit the enzyme. Studies of mechanisms of these inhibitory effects have already been described in the results section.

The Mg^{++}/ATP ratio of 1/1 which is necessary for maximum hydrolysis of ATP is probably required to keep free Mg^{++} and free ATP in low enough concentrations so they will not inhibit the enzyme.

The mechanism of ADP inhibition is not clear. ADP might compete with ATP for Mg^{++}, although the association constant of Mg ATP is larger (10^4) than that of Mg-ADP $(10^{3.11})$ (Chaberek and Martell, 1959). Kielly and Kielly (1953) have shown that the inhibition of mitochondrial ATPase by ADP cannot be overcome by increasing Mg^{++} concentrations, and they suggested that ADP or Mg ADP competes with the substrate Mg ATP for the active site. Speculations concerning the mechanism of ADP inhibition of the

ATPase activity of myometrial microsomes would not be
fruitful because of lack of data.

The mechanisms involved in excess Mg ATP and
excess ATP inhibition of the microsomal ATPase cannot
be specified from the data presented. Many enzymes
are inhibited by excess substrate, and we may speculate
that such is the mechanism of Mg ATP excess. The sub-
strate may overload the active site of the enzyme, and
prevent normal substrate enzyme complex formation
essential to substrate hydrolysis. (Dixon and Webb, 1964).

Excess ATP may bind to a site near the active site
and prevent the complex formation mentioned above. The
easiest explanation of the ATP protection experiments
to be discussed later, is that ATP binds to the enzyme
and alters its configuration. These interactions may
also inhibit the hydrolytic activity.

D. Effects of Ca^{++}

Ca^{++} has a dual effect on this enzyme. When Mg^{++}
is absent, Ca^{++} can activate the ATPase activities both
in mitochondria and microsomes (see results and Wakid,
1960). This same activity has been noted in rat liver
microsomes (Ernster and Jones, 1962) mitochondria
(Ulrich, 1966) and skeletal muscle microsomes (Duggan,
1964, 1965).

Part of the mechanism of action of Ca^{++} inhibition has been worked out for the microsomal ATPase of this study by comparing the calculated concentration of various components in the assay media to the enzyme activity (Table IX). It was found that the inhibition of the activity by Ca^{++} in the presence of Mg^{++} was correlated best with the concentration of the Ca ATP complex, even though the Ca ATP can be hydrolyzed. Ca ATP has recently been shown to be the inhibitory species when Ca^{++} inhibits the Na^+ + K^+ ATPase activity of red blood cells and liver microsomes (Epstein and Whittam, 1966), and rat liver mitochondrial ATPase (Ulrich, 1966). The extent of inhibition of Mg ATP splitting by Ca ATP is probably greater than suggested by the reduced rate of P_i formation, since Ca ATP hydrolysis may contribute much of the P_i produced (Figure 19).

In microsomal fractions from rat myometrium, Ca-ATP may compete with Mg ATP for the same enzymatic site. Inhibition by Ca ATP could not be explained if one assumed the presence of two separate enzymes, a Ca ATPase and a Mg ATPase, each interacting with its own substrate. If this assumption were made, ATP would be the limiting factor.

Assuming 2 such separate enzymes, the two activities Ca ATPase and Mg ATPase should be additive, as long as ATP is readily available. The activities

at relatively high ATP levels are not additive for the microsomal fraction.

As Ulrich found, this present work has demonstrated a possible inhibitory effect of free Ca^{++} itself, when present in high enough concentrations. Ulrich has suggested that Ca^{++} can compete with Mg^{++} for a site on the enzyme.

Data available do not permit speculation as to the mechanism of inhibition by free Ca^{++}.

The results obtained to date may be consistent with the existence of two enzymes if the Mg ATPase can be inhibited by Ca ATP, and the Ca ATPase cannot be inhibited by Mg ATP. Further studies showing that neither enzyme can be isolated or selectively inactivated are required before it can be concluded that only one enzyme is responsible for the observed activities.

E. Effects of Zn^{++}

The effects with Zn^{++} on the fresh microsomal fraction are very similar to those with Ca^{++}. This ion can also partially replace Mg^{++} in its absence and inhibits ATPase activity in its presence.

The relative abilities of the ions to act as activators in the absence of any other ions is $Mg^{++} > Ca^{++} > Zn^{++}$. The activities were Mg ATPase, 80 μmoles

P_i/mg. N/5'; Ca ATPase, 41 μmoles P_i/mg. N/5'; and Zn ATP-ase, 29.7 μmoles P_i/mg. N/5'.

This cannot be related to the affinities of these ions for ATP. The respective values of association constants for Me^{++} ATP are as follows (the values are in moles^{-1}, and are the average values taken from the book by Martell and Sillén, 1964): Zn ATP, $10^{4.85}$; Mg ATP, $10^{4.2}$; Ca ATP, $10^{3.74}$. If ability to form the metal complex were the regulator of enzyme activity, Zn^{++} would activate ATPase the most.

The slow hydrolysis of Zn ATP, and its ability to compete with Mg ATP for the enzyme's active site may account for its ability to inhibit hydrolysis of this substrate. This is one of the mechanisms proposed for Ca ATP hydrolysis, and, as in that case, other hypotheses have not been excluded.

F.   Effects of Ouabain

The cardiac glycoside ouabain has been shown to inhibit the Na^+ + K^+ activation of membrane (microsomal) ATPase (see introduction). It has not been shown to act on microsomal ATPase in the absence of these ions (i.e. it does not inhibit the basic Mg ATPase activity). However, in myometrial microsomes, ouabain does inhibit the basic Mg ATPase activity in the absence of any added Na^+

and K^+.

In the fresh microsomal fraction, ouabain inhibited the enzyme from 10 to 30% irrespective of the presence or absence of Na^+ or $Na^+ + K^+$ (Table XVA, Table XVIII). However, Table XXV shows that ouabain seems to inhibit more activity of stored microsomes in the presence of Na^+ stimulation, although some inhibition is still seen in the absence of Na^+ (about 12%). An inactive preparation, hexahydroscillaren, had no effect on the enzyme activity.

G. Comparison of Microsomal and Mitochondrial ATPases

There are similarities between the fresh ATPases of the microsomal and mitochondrial fractions of this tissue.

1) They both require a divalent cation for ATPase activity.

2) The optimal Me^{++}/ATP ratio is 1/1 (Me^{++} = Mg^{++} or Ca^{++}).

3) Mg^{++} is a better activator than Ca^{++}.

4) Both have very low substrate specificity (using Mg^{++}/NTP ratio of 1/1).

The following differences exist as well.

1) The mitochondria have no ouabain sensitive ATPase activity, but the microsomes do.

2) The microsomal ATPase can be stimulated by Na^+ after specific treatments and the mitochondrial ATPase cannot.

No conclusions can be reached regarding the existence of different enzymes in each fraction, or the existence of the same enzyme whose properties may be altered in one fraction and not the other.

H. Preincubation Studies of Fresh Microsomal Mg ATPase

Interesting effects regarding this enzyme activity were found when aliquots of this fraction were preincubated with NEM, urea, NaF and ATP. If the enzyme was preincubated with one of the first three compounds, subsequent assay for Mg ATPase showed a decreased activity (Table XXXVI). When these compounds were preincubated in the presence of 5 mM ATP, their respective inhibitory effects were prevented or diminished. In the case of urea, enzyme protection by ATP was essentially complete while protection against NEM and NaF was about 75%. (Preincubation was essential for protection, since these three compounds inhibited activity when added to an assay medium with 5 mM ATP. For information regarding the preincubation and assay procedures, the reader is referred to Tables XXXVI and XXXVII). Studies were not made with altered ATP concentrations.

Skou and Hilberg (1965) demonstrated similar effects, but also showed that such treatment increased the $Na^+ + K^+$ sensitivity of the ATPase (i.e. an increased activity ratio). This latter effect was not demonstrable in this tissue, although under certain conditions NaF in the assay medium did elicit some Na^+ activation.

The preincubation results described above cannot be easily explained. The three different compounds must be altering the protein configuration, and this alteration can be prevented by ATP. This implies either that all three compounds must have a common site of interaction or elicit a similar configurational change, which may be blocked by the nucleotide, or that combination at several sites of interaction or several configurational changes may be prevented by ATP interacting with the protein. The accepted mechanism of NEM action on proteins is that it combines with free sulphydryl groups, and of urea, that it interferes with hydrogen and possibly hydrophobic bonding of proteins. NaF has not been shown to act in either of these ways, and its specific effect on aldolase (Gemmill, 1939) and succinic oxidase (Slater, 1952) are as yet only partially explained (Simons, 1965).

Since these 3 agents presumably all act differently, ATP probably protects against interaction at several sites, or against configurational changes of diverse

origin. This may imply an ATP effect on protein configura-
tion which may account for nucleotide inhibition of ATPase
as well as enzyme protection, since Mg^{++} was not neces-
sary in either case (see page 166 of discussion).

V. $\underline{Na^+\ Activation\ of\ Microsomal\ ATPase}$

Throughout this work, no activating effects of either
Na^+ or K^+ or both have been found in either the fresh
microsomal or mitochondrial fraction. The only effects
noted were inhibitory effects of Na^+ on fresh microsomal
and mitochondrial ATPase activity in the presence of 50
mM Tris buffer (Tables X and XI). When 50 mM histidine
buffer was substituted for Tris, no Na^+ inhibition or
activation was noted in either fraction, and enzyme
activity was somewhat greater. Histidine may cause a
greater activity by interacting with the enzyme at or
near the site at which Na^+ exerts its inhibitory action.

Ulrich (1963) has shown that Na^+ can inhibit the
ATPase activity of rat liver mitochondria. The Na^+ in-
hibited ATPase of myometrial microsomes may be mito-
chondrial in origin, and represents the mitochondrial
contamination discussed earlier. Alternatively both
fractions may have similar ATPase activities.

After certain treatments of the fresh microsomal
ATPase, Na^+ activation was obtained in rat myometrial

microsomes. These treatments were:

1) DOC

2) Storage

3) DOC plus storage

4) NaN_3

5) NaF

6) Urea

It should be noted that in all cases increased activity ratios were produced by a faster decrease of the Mg ATPase activity than of the $Na^+ + K^+$ Mg ATPase or Na^+ Mg ATPase. Thus the activity ratio would increase as the denominator decreased faster than the numerator $(\frac{Mg+Na^+}{Mg})$.

Do all these procedures elicit Na^+ stimulation by a common effect? Samaha and Gergely (1966), Auditore and Wade (1964) and Schwartz and Laseter (1964) have all suggested that the use of NaN_3 increases $Na^+ + K^+$ activation by inhibiting only the mitochondrial Mg ATPase, which is not stimulated by these ions.

The argument that NaN_3 inhibition of a mitochondrial ATPase unmasks a $Na^+ + K^+$ activation requires that this latter activity always be present. If NaN_3 were acting by selective inhibition of mitochondrial ATPase (insensitive to Na^+ and K^+) then the absolute amount of Na^+ activated ATPase would be unchanged by NaN_3, and the relative

amount of this ATPase would be increased in proportion
to the NaN_3 concentration and mitochondrial ATPase in-
hibition. Thus even in the absence of NaN_3, some Na^+
+ K^+ activation should be evident, and the addition of
NaN_3 should increase the proportion of the Na^+ + K^+
dependent enzyme already present, but not the absolute
amount of such an enzyme. This is true for a micro-
somal fraction of human skeletal muscle (Samaha and
Gergely, 1966), rabbit kidney mitochondria (Auditore
and Wade, 1964) and guinea pig cardiac muscle micro-
somes (Schwartz and Laseter, 1964), but not for rat
cardiac muscle microsomes (Schwartz, 1962) and rat
myometrial tissue (Table XXXI). In the latter two
cases, there is no Na^+ or Na^+ + K^+ activation present
in the fresh untreated microsomes.

As mentioned earlier, Ulrich has shown Na^+ inhibi-
tion of rat liver mitochondrial ATPase. However,
Peña-Diaz et al (1964) using mitochondria from the same
source were unable to demonstrate any such effect with
intact mitochondria. If, however, the mitochondria were
disrupted or altered by DOC or sonication, larger activ-
ity was noted in the presence of K^+.

Fresh mitochondria of rat myometrial tissue showed
no Na^+ inhibited ATPase activity with histidine buffer
in which Na^+ activation of microsomes could be demon-
strated. This result is not in accord with the hypo-

thesis that elimination of a Na^+-inhibited mitochondrial ATPase by NaN_3 accounts for the Na^+ activation observed. However, the possibility exists that submitochondrial particles in the microsomal fraction are Na^+ inhibited in Tris buffer.

Ageing might also induce Na^+ activation by unmasking an activity present from the outset, but obscured by Na^+ inhibited mitochondrial ATPase activity. Na^+ activation of aged microsomal fractions is much larger in histidine buffer than in Tris buffer (Figure 27), and no Na^+ inhibition of fresh mitochondrial or microsomal ATPase was observed with histidine. Aged mitochondria demonstrated no Na^+ inhibitory or activating effect with histidine or Tris, and it seems unlikely that decreased amounts of Na^+ inhibited mitochondrial ATPase activity has much to do with the effect of ageing in this tissue.

The ability of ouabain to inhibit the ATPase activity of fresh microsomal material might also be attributed to the masked Na^+ activated ATPase. The unmasking hypothesis could not account for the appearance of more Na^+ activated enzyme on ageing than ouabain inhibited before ageing. Conversion of Na^+ independent or inhibited ATPase activity to Na^+ dependent activity is the most reasonable explanation.

$Na^+ + K^+$ effects on rat liver microsomes may be explicable by the hypothesis of unmasking the ATPase effects. Ernster and Jones (1962) and Schwartz (1964) were unable to demonstrate any $Na^+ + K^+$ effects in fresh microsomes. However, the latter worker showed that ageing at $-5^\circ C$ demonstrated Na^+ stimulation. Destruction of a Na^+ inhibited mitochondrial ATPase by ageing may have occurred. Ulrich (1963) has shown Na^+ and K^+ inhibition of fresh rat liver mitochondria, but studies on aged rat liver mitochondria for these purposes have not been carried out.

Skou (1962) showed that it was necessary for DOC to be present in the isolation medium and assay medium to get the maximum activity ratio in brain and kidney microsomal preparations. His suggestion was that DOC had at least two effects, one to decrease the activity of the Mg^{++} activated, $Na^+ + K^+$ insensitive enzyme, and the other to facilitate the separation of the above enzyme from the $Na^+ + K^+$ activated Mg ATPase. This explanation would necessitate the presence in the untreated fraction of some Na^+ activating effect or the masking of this activity by a Na^+ inhibited ATPase.

The effect of urea as a protein denaturing agent has long been known. Its addition to the isolating

medium raises the activity ratio of subsequently assayed microsomal ATPase. It may well act similarly to DOC.

The addition of 10 mM NaF to the assay medium also elicits Na^+ activation. This compound must have an effect on the Mg ATPase when added directly to the assay medium since preincubation of the microsomal fraction does not give the same effects.

Thus there is no evidence that inhibition of mitochondrial or other Mg ATPase activity by these treatments enhances the Na^+ activation. The only other suggestion left is that such treatments alter some of the Mg ATPase activity, converting it to a Na^+ sensitive activity.

Thus it seems proper to conclude that the mitochondrial ATPase explanation for Na^+ activation of treated rat myometrial homogenates is not valid.

Furthermore, Schwartz (1965b) has shown that histones preferentially inhibit the Mg ATPase ($Na^+ + K^+$ insensitive) activity of guinea pig heart microsomes, but that the compound stimulates mitochondrial Mg ATPase activity (1965a). No increase in the activity ratio in rat myometrial tissue was noted with histone treatment. All ATPase activity of microsomes and mitochondria was depressed. The histone effect must be unrelated to any of the six effects listed here.

At this point, the effects of ouabain on the aged Na^+ acti-
vated ATPase activity should be mentioned. If ageing were ac-
ting by selectively inactivating a ouabain insensitive ATPase,
the proportion of ouabain sensitive activity would increase and
the absolute amount would be unchanged. 1 mM ouabain inhibited
stored Mg ATPase in the absence of Na^+ (Table XXV). However, the
absolute amount of ouabain inhibition is larger in the presence
of Na^+ activation than in its absence, and the relative amount
of ouabain inhibition of the activity remained the same. There-
fore, ageing actually converts ouabain insensitive material to
ouabain sensitive material. Furthermore, the absolute increase
in ouabain sensitive material was about 30% of the increase of
Na^+ activated material $(\frac{6}{20})$, indicating that not all the Na^+
activation was ouabain sensitive, but 30% of it was (compare
this value to the 10-15% inhibition of Mg ATPase by ouabain in
the absence of Na^+, Table XXV).

Thus ageing causes the appearance of a Na^+ activated ATPase,
a larger portion of which (30%) is ouabain inhibited than of
fresh ATPase.

It should be noted that the very high K_M value for Na^+ in
aged microsomes (20 mM) would indicate that the ion is bound
less readily to this enzyme than to other ATPases, and that more
of the ion is necessary to induce the conformational change neces-
sary for activation. This may be directly related to the fact
that no K^+ activation site is present on this enzyme. It is
known that on other transport ATPases, two sites are available,
a Na^+ selective one and a K^+ selective one. Assume that in this
tissue the two sites are Na^+ selective only, and site 1 alters

the affinity of site 2, and the K_M for site 2 is much larger than the K_M of site 1. Thus much Na^+ could be bound at site 1 without causing a change in activity. A change would occur only when Na^+ began to site at site 2, for which Na^+ binding at site 1 need be complete. No Na^+ binds at site 2 until site 1 is saturated. Site 1 does not affect the enzyme activity, it only affects the site 2 Na^+ binding, which in turn does cause activation of the enzyme.

VI. Methods not Showing Na^+ Activation

A brief discussion of the various procedures that did not show Na^+ activation is in order (see section VIC of results).

The reasons why these procedures failed to give Na^+ activation are unknown. However, reasons for the use of the treatments should be mentioned.

Dialysis and microsomal washings were performed for the same reason. Järnefelt (1964) has suggested that the presence of relatively large amounts of Na^+ and K^+ complexed by the microsomal fraction from rat brain prevents any effect of adding more of these cations to the preparation. Thus these procedures were carried out in order to lower Na^+ and K^+ concentration complexed to the enzyme. Järnefelt resuspended his microsomal preparation in varying concentrations of DOC to achieve this end. In my experiments, removal of Na^+ and K^+ by washing and dialysis was ineffective in producing Na^+ activation. DOC under certain conditions did produce Na^+ activation, but whether this was a consequence of Na^+ removal per se is unknown. If so, removal of Na^+ by other procedures must result in other changes which interfere with Na^+ activation.

The addition of EDTA to the isolating medium or to the final microsomal assay was to see if chelation of any

divalent cations complexed to the enzyme might alter its activity. The hypothesis was that endogenous divalent cations might have had a stabilizing effect on the Mg ATPase but not on the $Na^+ + K^+$ (or Na^+) ATPase, or that such cations might selectively inactivate the $Na^+ + K^+$ stimulated ATPase.

Heat treatment ($65^\circ C$ for 10 minutes, Somogyi, 1964) was used successfully with rat brain microsomes to increase $Na^+ + K^+$ activation of ATPase. The mechanism of its effect is unknown. Heating destroys the Mg ATPase activity faster than the $Na^+ + K^+$ activity (i.e. increases the activity ratio).

Sucrose density subfractionation of the microsomal fraction was attempted on the supposition that the Mg ATPase might be physically separable from the Na^+ stimulated ATPase.

Sonication of red blood cell ghosts has been shown to produce an ATPase activity which could be activated by either Na^+ or K^+ alone (Askari and Fratantoni, 1963).

Different media for isolation of microsomes were tried because each one had been used successfully in isolating fractions from other tissues containing $Na^+ + K^+$ activated ATPase.

The reasons for use of heparin, histone, NaI, isolation with metabolic inhibitors and ions and different centrifugal forces have already been discussed briefly

(see results, section C).

VII. Comparison of ATPase Activities of the Four Different Preparations

The ATPase activity of rat myometrium has been studied on 4 different preparations.

1) intact tissue

2) microsomal fractions

3) plasma membrane preparation

4) total homogenate

After certain treatments of the microsomal fraction, Na^+ activation could be seen. Ouabain inhibited the ATPase activity of this fresh preparation, both in the presence and absence of Na^+, and in the stored fraction there seemed to be a relation between Na^+ stimulation and ouabain inhibition, since Na^+ activated ATPase activity (the actual Na^+ activation per se) could be partially inhibited by ouabain. Under no conditions could any K^+ stimulation alone, or with Na^+ be demonstrated.

The membrane preparation showed Na^+ activation which appeared to be related to the ouabain inhibition, since most of the Na^+ activation was inhibited. Again, no K^+ activation, either alone, or with Na^+ could be

demonstrated.

The ATPase of the total homogenate was shown to be Na^+ stimulated, and this Na^+ stimulation appeared to be related to ouabain inhibition. No K^+ activation was observed.

It has been shown that the Na^+ activated ouabain inhibited ATPase is not lost in the nuclear fraction, since the activity can be demonstrated in the fresh nuclear supernatant, and aged mitochondrial supernatant. Furthermore, no treatment of the nuclear fraction could give Na^+ or K^+ effects.

Somehow during the separation of the nuclear supernatant to the mitochondrial fraction and mitochondrial supernatant, the Na^+ and ouabain sensitivities are altered. Although no specific conclusions can be reached at this time the fact that certain treatments can bring back the Na^+ and ouabain sensitivity to the microsomes, and the indication that nothing is lost to the mitochondria leads to the suggestion that material is gained from the mitochondria and alters the enzyme. The various treatments somehow cancel the alteration allowing the activity to return. The data obtained allow no further speculation.

The intact tissue showed no ouabain effect on the ATPase activity using either phosphate free Krebs or the total homogenate assay medium ($Na^+ + K^+$) as the suspending fluid. Na^+ appeared to have no effect on the ATPase activity of the intact tissue suspended in total homogenate media or Na^+ free Krebs. The presence of other surface enzymes on the muscle should not be overlooked. Until a specific study of nucleotide phosphokinases (NTP kinase) is completed in this preparation, the relation between ATP splitting by intact tissues and by microsomes will remain obscure.

Ouabain had a definite effect on all the preparations except the intact tissue. This leads to the hypothesis that ouabain may be acting to inhibit the ATPase on the inside of the cell. The three preparations in which ouabain inhibited the Mg ATPase activity were all preparations in which cells were largely disrupted and the compound would have free access to its proposed site of action. However, the following discrepancy arises. If ouabain does not inhibit the ATPase of intact tissue, why does it cause downhill ion movements in the same

preparation (Daniel, 1963)? The answer may be that the ATPase activity measured in intact tissue uses externally added ATP as its substrate, while internal ATP is used as the energy source for active transport in the intact cell.

If such were the case, the external ATPase activity of intact cells would be unrelated to active cation transport and might be expected to be ouabain insensitive. This activity might also be expected to be isolated along with any transport ATPases in membrane preparations and should contribute to the Mg ATPase and Ca ATPase activities.
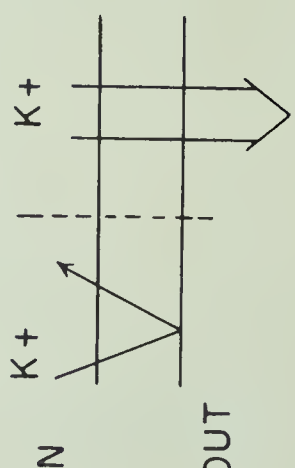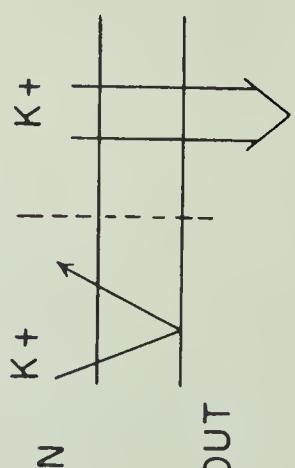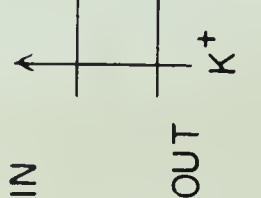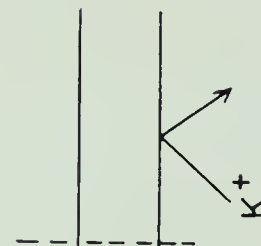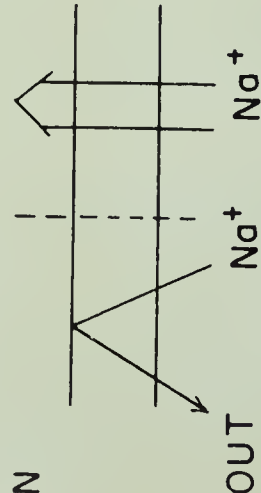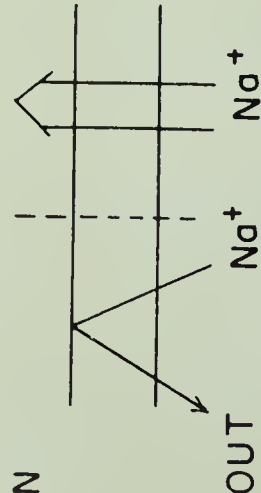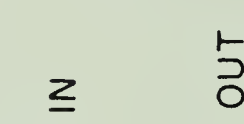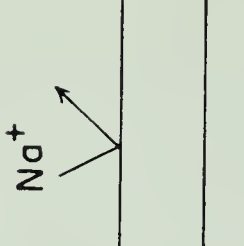
It has been shown that ouabain does not alter ATP, ADP or AMP levels in uterine tissue (Daniel et al, to be published) so its activity must be on the transport process itself, and not on energy supply.

The effect of this compound on the fluxes of other tissues, using the red blood cell as prototype leads to decreased K^+ influx and decreased Na^+ efflux. However, in rat myometrium the flux result of ouabain addition is increased Na^+ influx and probably increased K^+ efflux (Daniel, to be published). These differences may be seen in the accompanying diagram.

VIII. Conclusions

It is believed that active Na^+ exclusion is present in this tissue, and the demonstration of an ATPase with unusual cationic characteristics (i.e. no K^+ activation, only

DIFFERENCE OF ION FLUXES BETWEEN RAT UTERUS AND RED BLOOD CELLS.

Na^+ activation) is not wholly unexpected.

Although no model for active ion transport in the rat uterus will be proposed, it is obvious that because of the cationic effects on the uterine microsomal ATPases, and since relative values of intracellular Na^+ (18 meq./kg. cell water) and K^+ (190 meq./kg. cell water) are similar to those found in numerous other tissues necessitating that an active transport mechanism is in operation, the classical model for active ion transport cannot be applied to this tissue. A model must fulfill the following requirements.

1) Na^+ and K^+ must move in roughly reciprocal amounts when active ion transport is inhibited.

2) Downhill ion movements are effected primarily by increased Na^+ influx and K^+ efflux when brought about by ouabain or K-free solutions.

3) Downhill ion movements are effected by the same mechanism plus decreased Na^+ efflux and K^+ influx in the presence of iodoacetate (and lowered internal ATP).

4) Na^+ activation of ATPase (presumably by formation of E~P and breakdown to E + P_i) occurs independent of K^+.

5) Na^+ stimulation need not be completely inhibited by ouabain.

6) Ouabain may act at the inner surface of the cell membrane.

Points 4 through 6 are a direct result of findings in this thesis and deserve some comment.

4) The lack of any K^+ effect on the ATPase activity necessitates Na^+ activation of both the formation and breakdown of a phosphorylated intermediate (see introduction). It is difficult to conceive of a mechanism of active cation transport involving ATPase without this intermediate.

5) The fact that Na^+ activation may not be completely inhibited by ouabain may be an artefact of the microsomal fraction in view of the glycoside's effect on the Na^+ activation of total homogenates and plasma membrane. The Na^+ activation (maximum of 60%) is very small when compared with other tissues (Skou, 1965).

6) Ouabain's suggested site of action at the inner surface may be an artefact of the intact tissue preparation. Experiments elucidating all related surface enzymes present would have to be carried out to clarify the situation. The inside site of action could not be responsible for the relative insensitivity of the tissue to ouabain, since the $Na^+ + K^+$ ATPase of other rat tissue (cardiac) is also relatively insensitive to ouabain and the glycoside presumably acts on the outside in that tissue. The insensitivity of rat myometrium is not peculiar to this tissue, but to other rat tissues as well. A more general mechanism should be

used to explain this phenomenon.

(It should be noted that recently, Czerwinski et al (1965) have demonstrated an ATPase from the rat erythrocyte, which is Na^+ activated. This activation was completely inhibited by the glycoside scillaren (concentration not given) but the enzyme was not stimulated by K^+.)

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